Proflavine-Dependent Modification of Macromolecule in the Presence of Transition Metal ions

Abstract

For Thesis Submitted for the Award of the Degree of

Doctor of Philosophy

In

Biochemistry

By

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Aligarh Muslim University
Aligarh (INDIA)

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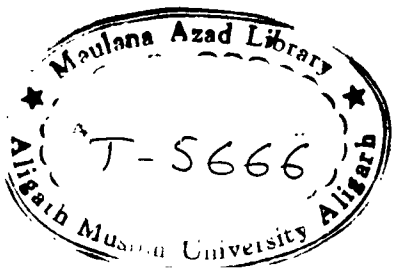
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Proflavine, 3,6-Diaminoacridine, is an acridine dye having a flavine nucleus. It is a known antibacterial agent and strong DNA intercalating agent. Several investigators were using it as fluorochromes because of its high quantum efficiency, which made it especially useful for automated cell analysis. Associations of proflavine with DNA have been reported to give specific effect, like frameshift mutation during in vitro replication of single-stranded DNA template. Proflavine is known to be structurally similar to tacrine and quinacrine, drugs approved for the treatment of Alzheimer's disease and malaria, respectively. Studies using human hepatocytes with proflavine and tacrine show subcellular changes and mitochondrial dysfunction. A toxic role of oxidized proteins, rather than oxidized lipids, has been recently proposed in the etiology of Alzheimer's disease.

Proflavine is a photodynamic agent that is known to target DNA as well as other biomolecules. Photoactivation of proflavine is known to generate different type of free radicals such as singlet oxygen (O_2^1), hydroxyl radical ($^{\cdot}OH$) and peroxide radical. In the present study we have shown that proflavine alone upon photoillumination can generate reactive oxygen species (ROS). The addition of various free radical scavengers suggested that the triplet oxygen (O_2^3) is the major species generated in the reaction in addition to superoxide anion ($O_2^{\cdot-}$), O_2^1 and $^{\cdot}OH$. Absorption spectra of proflavine shows that it undergoes degradation upon

photoillumination. Cu(II), when present in the reaction mixture together with proflavine partially restores both the proflavine peaks, while presence of protein did not help or prevent photodegradation of proflavine. Furthermore, presence of double stranded DNA, single stranded DNA and RNA all caused red shift in the peak of proflavine which is indicative of binding with the double stranded DNA, denatured DNA and RNA. However, the shift was maximum with double stranded DNA. Moreover, after four hours of incubation in fluorescent light the double stranded DNA was able to inhibit photodegradation of proflavine completely. Our observation indicates that the photogeneration of $O_2^{\cdot -}$ from proflavine was faster in presence of dsDNA. We have proved also that the presence of guanine residues in DNA is essential for the stimulatory effect of dsDNA on $O_2^{\cdot -}$ generation by proflavine in visible light.

Proteins are the key targets of ROS leading to their oxidation that have been shown to control cellular remodeling and growth. A survey of literature revealed that interaction of proteins with oxygen free radicals both in vivo and in vitro results in enhanced hydrophobicity due to modifications of amino acids. Proflavine derived ROS were found to damage the protein molecule (BSA) and this effect was enhanced when the divalent metal ion, Cu(II), was included in the reaction. Proflavine treated BSA with or without Cu(II) upon photoillumination was found to become resistant to proteolysis as is evident from decreased production of acid soluble material upon trypsin treatment. In the absence of light, however such effect was not seen. The SDS-PAGE profile of BSA after exposure to photoilluminated proflavine showed significant degradation. Presence

of Cu(II) resulted in concentration dependent enhancement of protein degradation to smaller fragments.

We have shown that ·OH is involved in degradation of BSA by photoilluminated proflavine as is evident from the use of various free radical scavenger. However, there is strong evidence for the formation of O^1_2, O^3_2 in the reaction mixture. Bathocuproine, a specific Cu(I) sequestering agent, when present in the reaction mixture containing Cu(II), was found to inhibit the protein degradation, showing that Cu(I) is an essential intermediate in the reaction.

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loss of activity of invertase after incubation with proflavine and light have taken longer time than the case with trypsin. Surprisingly, electrophoretic profiles of these two enzymes have shown that modification of invertase by the photoilluminated proflavine was faster as compared with trypsin. The intensity of the major band of trypsin requires longer time of incubation with proflavine and light than that of invertase. This could be due to the formation of bi-tyrosine crosslinks in trypsin as suggested by other investigators. Results obtained with various scavengers of active oxygen species strongly suggest that singlet oxygen, triplet oxygen and hydroxyl radical are predominantly responsible for the damage in the both enzymes. A probable mechanism of photoexcitation of proflavine leading to generation of various active oxygen species in presence of Cu(II) has also been proposed.

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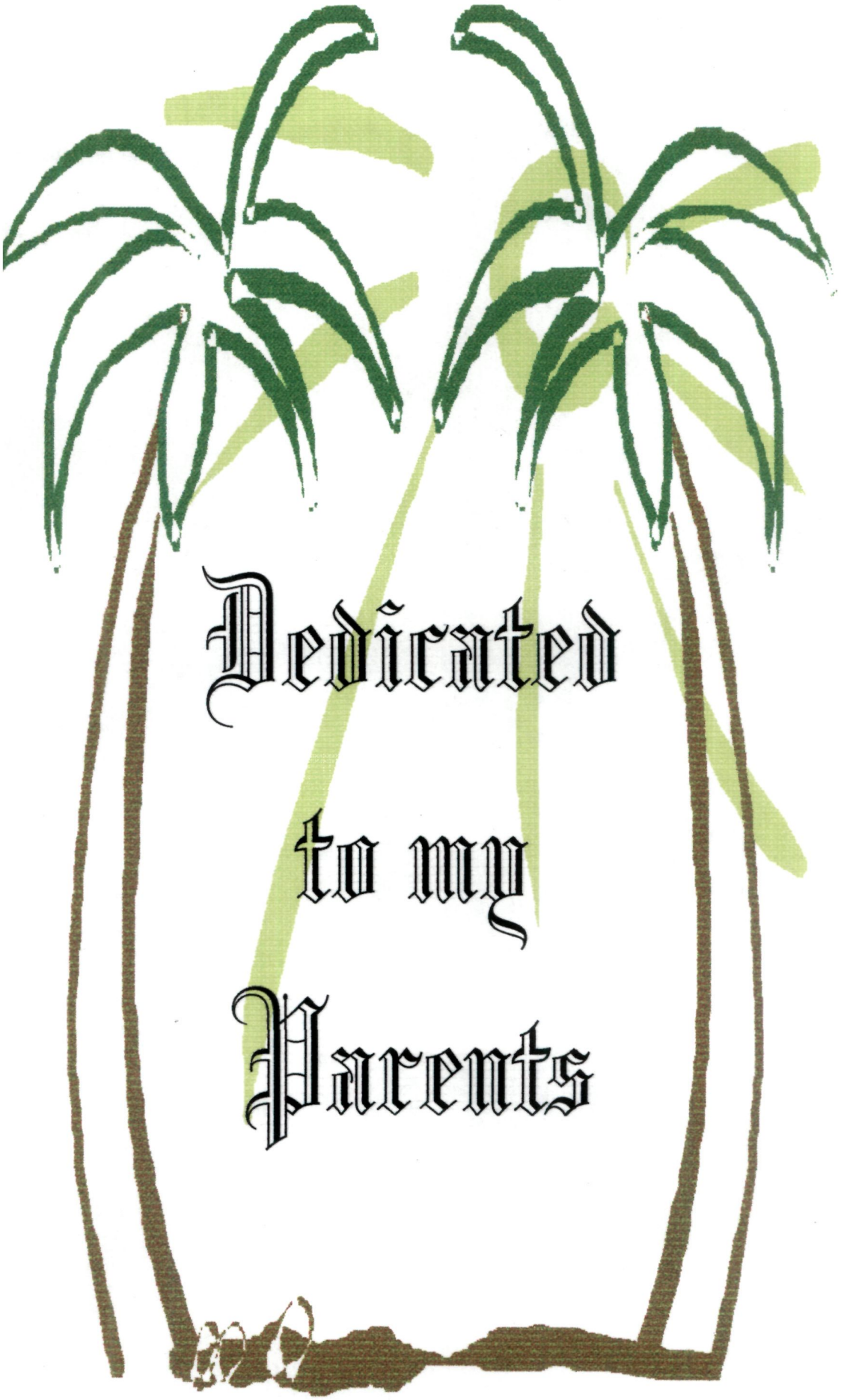
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Parents

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ALIGARH MUSLIM UNIVERSITY
ALIGARH—202 002 INDIA

REF. NO.....

DATED Oct 5th 2007

CERTIFICATE

I certify that the work presented in this thesis has been carried out by ***Mr. Mansour K.M. Gatasheh*** under my supervision. It is original in nature and has not been submitted for any other degree.

Dr. Imrana Naseem
(Reader)

Acknowledgement

To the Almighty I bow my head in humility and reverence for having gifted man with the faculty of reasoning and a spirit of discovery.

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Mansour K.M. Gatasheh

8.10.2001

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CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

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Introduction

Acridine

The discovery of a basic material in the anthracene fraction of coal tar was announced by Graebe and Caro in 1870. On account of its acrid smell and irritating action on the skin and mucous membrane this new substance was called "Acridine" (acris = sharp, or pungent). Then a series of salts were prepared. A most characteristic property of acridine, and many of its derivatives, is the fluorescence which they exhibit in dilute solution (Acheson, 1973).

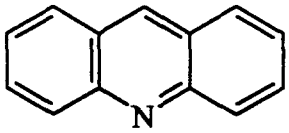
Ehrlich later discovered the therapeutic property of acridine, acriflavine and proflavine were introduced as antibacterial agents during first world war, when there was an increased demand for active antibacterial agents, which would prove less toxic than known substances to the deep tissues which become exposed in wounds. Aminoacridines penetrate tissues readily, not so readily as many sulphonamides, but better than the triphenylmethane dyes such as crystal violet. It was found that when a small mammal was injected with an aminoacridine, and the tissues quickly frozen and sectioned one hour later, the acridine is seen to have been taken up by many kinds of cells and concentrated in the nuclei. However the cells of the central nervous system did not absorb the dyes. There was little or no further clinical experimentation with acridines until the First World War (1914-1918), when acriflavine and proflavine were introduced as local antibacterial agents, in which there was an increased demand for active antibacterial agents, which would prove less toxic than known substances to the deep tissues which become exposed in wounds. (Alberts, 1966)

Photochemistry of Acridines

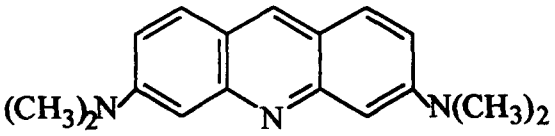
Fluorescence is generally observed in those organic molecules which have rigid framework and not many loosely coupled substituents though which vibronic energy can flow out. In general, some substituents in these molecules enhance the fluorescence and they are known to be electron donors such OH, NH_2, etc, which enhance the transition probability or intensity of colour, e.g. acridine and acridine orange as given in figure 1.

For designating energy levels of these compounds, the electronic states are

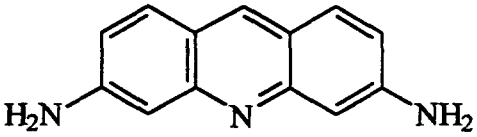
Figure 1. Diagrams of three acridine with different substitution have difference in fluorescent emission.



acridine
(non fluorescent)



acridine orange
(fluorescent)



Proflavine
(fluorescent)

expressed in terms of the initial and final orbitals involved in a transition. An electron from any of the occupied orbital can be promoted to higher unoccupied level on absorption of appropriate radiation. On an electron promotion, the *energy state* of the molecule changes, and may have singlet or triplet character. (Rohatgi-Mukherjee, 1986).

Action of acridines

Interaction with Nucleic Acids: The nature of the interaction of acridine derivatives, especially aminoacridines, with nucleic acids has attracted increasing attention since their earliest use as cellular stains. The widespread biological effects of acridine derivatives gradually came to be connected principally with their ability to interact with nucleic acids, but interest in this interaction was heightened when the mechanism of the binding processes involved was understood. The binding ability to nucleic acid depends on the basicity of the acridine which will cause a marked increase in intrinsic viscosity and a decrease in sedimentation rate of DNA, and this indicates an increase in counter length of the DNA double helix (Acheson, 1973).

Action on Enzymes: Interest in the biological activity of the acridines has centered on their antibacterial, antimalarial and mutagenic properties. Apart from a few isolated instances, little interest has been taken in their action on enzymes until the last 20 years or so. The interest in its action on enzymes was generated due to our increased understanding of cellular metabolism, as reaction of acridines with enzyme system usually resulted in the inhibition of the enzyme. The inhibition of RNA synthesis in rat liver mitochondria by low concentration of acriflavine revealed that mitochondrial DNA is involved in the process (Acheson, 1973).

The Antibacterial Action: An interest in the effect of acridines as wound disinfectants was developed since its antibacterial effect was stronger, in contrast to many other substances and was shown to be retained in the presence of body fluids and pus. Both proflavine and 3,6-diamino-10-methylacridinium chloride became widely used for this purpose. Some pathogen was found to be resistant to acridines (Acheson, 1973).

Proflavine

3,6-Diaminoacridine, having a flavine nucleus, as shown in figure 1, is a known antibacterial agent since Browning's work in 1913, but it is only in later years that its superiority over acriflavine was recognized. It is soluble in 300 parts of water or 35 parts of glycerol, a saturated solution in water is deep orange in color and gives a green fluorescence when freely diluted. Both proflavine and acriflavine were used as wound disinfectants during world wars. The application of not more than 0.5 g of powdered proflavine at a time was recommended, a treatment that gave good results in a number of intractable mixed infections (Albert, 1966).

Observation of Singh-Ranger and co-worker (2001) have presented a case where contact dermatitis in response to proflavine developed after pinnaplasty in which usually the proflavine allergy is uncommon. Proflavine, for umbilical cord care in the umbilical cord separation time was still in use (Hsu *et al.*, 1999).

The acridine dyes proflavine and acriflavine were applied as fluorochromes, by several investigators in the past because of their high quantum efficiency, which made them especially useful for automated cell analysis. It can be used routinely in clinical laboratories because this single stain could help in differentiation of leukocytes as well as counting of reticulocytes without altering the cells morphology (Sagawa and Tatsumi, 1997). The binding of proflavine to human adult hemoglobin in ferrous state will cause the X-band appeared in EPR spectrum to displays the characteristics of T-state of the ligated tetramer. In parallel, oxygen affinity for the deoxygenated derivative of ferrous human adult hemoglobin decreases in the presence of proflavine (Ascenzi *et al.*,, 1999). Recently the investigation made on acridines, especially proflavine, is its action on DNA and proteins (Revich and Ripley, 1990; Conti, 1998).

Several investigators studied the binding of proflavine and other acridines such as acriflavine on native denaturated unhydrolyzed DNA in solution. Proflavine were found to bind best to alternating purine-pyrimidine sequences regardless of their nature, the drug is thought to exert its biological action mainly by binding to DNA (Bailly *et al.*, 1992). In neutral aqueous solutions, proflavine exhibits a strong absorption maximum at 444 nm, and upon addition of DNA, a pronounced red shift in the proflavine absorption maximum to 460 nm occurs, that is indicative of the intercalation

of proflavine molecules (Marini and Caplow, 1971; Georghiou, 1997). Studies using CD spectroscopy showed that proflavine relax the superhelical organization of DNA, leading to the formation of a B-like structure without further structural changes as compared to other minor groove-binding drugs such as Hoechst-33258 which is reported to cause other structural changes (Krishna *et al.*, 1993). The introduction of this simple intercalator affects both the conformational features and dynamic properties of the oligonucleotide double helix, as both major and minor grooves became wider with the addition of the intercalating drug (Herzyk *et al.*, 1992). Water uptake accompany the complex formation indicate importance of water as thermodynamic participant (Qu and Chaires, 2001).

Association of proflavine with DNA have been reported to give specific effect, like frameshift mutation during in vitro DNA replication of single-stranded DNA template by the Klenow fragment of Escherichia coli DNA polymerase I. A novel inhibition of polymerization was found opposite to all pyrimidines in the template when proflavine template complexes were exposed for ten seconds in white light (Revich and Ripley, 1990), such frameshift is based on the hypothesis that the polymerase passes by a template base without copying it leading to deletion (Berman *et al.*, 1992). Thus, supporting the proposed mutagenic mechanism for proflavine-induced mutations in which frameshift is produced as a consequence of exonuclease or DNA polymerase activity at the 3` ends of nicks in the DNA is observed in experiment with thymidylate synthase gene of bacteriophage T4 (Brown *et al.*, 1993). Moreover, proflavine caused inhibition of transcription process, and did not cause displacement of the enzyme RNA polymerase from the promoter (Mazumder *et al.*, 1993, Kim *et al.*, 1998). Considerable interest has been focused recently on the role that some host transcriptional factors may play in the initial activation of human immunodeficiency virus (HIV-1) gene expression by interaction with the long terminal repeat of the integrated provirus (Haseltine, 1988), and proflavine was found to do both classical and threading intercalation with the TAR RNA of HIV-1 (Bailly *et al.*, 1996). Indirectly proflavine may cause gene expression of HIV-1 as proposed by Sylvie et al. (1995), intercalated proflavine is known to oxidize the DNA guanine upon irradiation with visible light.

Role of Proflavine in the Generation of Reactive Oxygen Species

Photochemical techniques are emerging as promising approaches for the sterilization of blood products, biological fluids, treatment of cancer and other diseases (Ben-Hur and Horowitz, 1996; Goodrich and Platz, 1997; Fisher *et al.*, 1995). These methods are based on the photodynamic effect in which biological target macromolecules are damaged by light-absorbing, photoexcited endogenous or exogenous chromophores. Single-photon absorption processes utilizing UV or visible light usually excite the photosensitizer molecules. However, the application of these techniques is often limited by a number of factors. For example, UV light, depending on its wavelength, can be intrinsically cytotoxic and/or mutagenic (Young, 1990). Furthermore, strong optical absorption and Rayleigh scattering effects can restrict the penetration depth of UV and visible light photons in biological tissues or fluids (Cheong *et al.*, 1990). The enhanced penetration depth of near-IR light into tissues and the lower extent of Rayleigh scattering offers self-absorption by photosensitizer molecules may limit the absorption of IR light into tissues (Wilson, 1989).

The use of multiphoton excitation of photosensitizer molecules has been recently explored with the goal of overcoming some of the limitations associated with single-photon excitation mechanisms (Leupold and Kochevar, 1997). The simultaneous absorption of two or more near-IR photons can provide an overall excitation energy equivalent to that of a single UV or visible photon.

Acridine dyes, espcifically proflavine, are photodynamic agents that are known to target DNA as well as other biomolecules (Kochevar and Dunn 1990; Kochevar and Buckley, 1990). Although the exact mechanism of photodamage to DNA initiated by proflavine are not well characterized. Whether its electronically excited state reacts directly with DNA via electron transfer and/or hydrogen atom abstraction mechanisms is not clearly understood (Kochevar and Dunn, 1990). Using 2`-deoxynucleotide-proflavine model systems, Georghiou (1977) found that only guanosine-5`-monophosphate give rise to a substantial quenching of the fluorescence. In contrast, all of the other nucleotides slightly enhanced the fluorescence of proflavine. Furthermore, an enhancement in the proflavine fluorescence decay kinetics is correlated with the G-C content of the DNA (Georghiou, 1977). These results indicate that guanine residues are responsible for the quenching of the fluorescence of proflavine when this molecule

forms complexes with DNA. Furthermore, because guanine is the most easily oxidizable nucleic acid base (Piette *et al.*, 1978), electron transfer from guanine to electronically excited proflavine molecule is likely to constitute the first step in the complex series of reactions that lead to DNA damage by strand cleavage (Piette *et al.*, 1981).

Earlier studies for the role of proflavine in inducing free radicals have been investigated by Piette and co-workers (Peitte *et al.*, 1978; Piette *et al.*, 1979; Piette *et al.*, 1981; Piette *et al.*, 1982). They compared different type of dyes, acridines, xanthene derivatives and sulphur-containing dyes. They analyzed the photosensitized induction of free radicals in calf thymus DNA at low temperature, and in the presence of oxygen, their results showed that the photoactivation of proflavine generate different type of free radicals such as singlet oxygen, hydroxyl radical and peroxide radical as detected by using Electron Paramagnetic Resonance, and the use of specific free radical scavengers. But these characteristic results were not obtained when proflavine or DNA were irradiated alone, nor when oxygen was absent. The induction of free radical from proflavine bound to DNA molecule can cause a single strand scission upon irradiation with visible light at high flounce rate as shown by agarose gel electrophoresis (Piette *et al.*, 1981). This is consistent with the hypothesis that a free electron is ejected during the excitation of bound proflavine by visible light. Both superoxide dismutase and ceruloplasmin decrease the e.p.r. signal observed in the reaction system suggesting that proflavine produces superoxide anions when complexed to DNA. Superoxide anion may be formed either by direct reaction between the electron ejected by excited proflavine and molecular oxygen, or by decomposition of the peroxide radical formed by the combination of a DNA base and molecular oxygen (Piette *et al.*, 1982).

Features of Proflavine

Recent investigations have shown that the presence of 6 μM intercalator proflavine can cause both direct single- and double-strand breaks in proflavine-supercoiled plasmid DNA complex (Shafirovich *et al.*, 1999). Wainwright et al. (1997) have showed that illumination at a light dose of 6.3 J/cm^2 resulted in considerable decrease in the minimum lethal drug concentration required, giving enhanced phototoxicity of proflavine against many strains type on the other hand, the aminoacridine and its derivatives were bactericidal at sub-millimolar levels, but were

photobactericidal at the micromolar level. Other researchers have on the negative aspect of photoilluminated proflavine. When irradiated proflavine oxidized the guanine residues of DNA, this will then cause signaling events which will be transmitted into the cytoplasm where the inactive NF-kappa B factor is present, p50/p60 subunits of NF-kappa B will be translocated to the nucleus leading to HIV-1 gene expression in cells surviving the treatment (Legrand-Poels *et al.*, 1995).

Furthermore, the recent observation of Singh-Ranger and co-worker (2001) which have present a case where contact dermatitis in response to proflavine developed after pinnaplasty, in which usually the proflavine allergy is uncommon. It may be possible because free radical production and disturbances in redox status can modulate the expression of a variety of inflammatory molecules, affecting certain cellular processes leading to inflammatory processes, both exacerbating inflammation and effecting tissue damage. It was recently reported that the activity of antioxidant enzymes in allergic patients, was higher than in non allergic controls giving indication for the involvement of free radical in allergic processes (Mates *et al.*, 2000). Other studies have suggested that drug allergy might come from the binding of drug non-covalently to MHC-peptide complexes and to T cell receptors, thereby able to stimulate T cells, hence inducing hypersensitive reactions (Pechler, 2001).

Do Proflavine Derivatives and Structurally Similar Drugs Have Similar Features like Proflavine?

Proflavine conjugate, such as 125iodoacetylproflavine, when added to human lymphoblastoid cells was readily taken up by the cells, localized in the nucleus, and was released rapidly following resuspension of the cells in fresh medium. This treatment caused an induced mutant fraction observed on Southern blot. When these results were compared with those observed with other intercalating drugs, the 125iodoaetylproflavine showed a reduced effectiveness per decay, perhaps related to the non-covalent nature of intercalator binding, resulting in reduced energy deposition in the DNA (Whaley *et al.*, 1990).

Studies have been carried out by synthesizing new polycyclic acridine derivatives and investigating their action on DNA. They were found to display a

stronger DNA-binding properties and high selectivity to [Poly (dA-dT)] polynucleotide than to [Poly (dG-dC)] polynucleotide as compared to proflavine (Gimenez-Arnau *et al.*, 1998). This indicate that the structure elements such as unsaturation or having different terminal group on the aromatic ring can cause a varied drug behavior. This may help in understanding the mechanism of action of different drugs on our body. Tacrine a drug used for the treatment of Alzheimer's disease was found to induce elevated serum aminotransferase levels, an indication of potential hepatotoxicty of tacrine. Similar cytotoxity was found to develope when proflavine and many more structurally similar compounds, were used. Studies have implicated the formation of reactive metabolites in the mechanism of hepatotoxicity (Monteith *et al.*, 1996).

Malaria caused by four species of plasmodial parasites (Wernsdorfer and Mc Gregor, 1988), is estimated to kill between 1.5 and 2.7 million people every year, with over 300 million currently infected. Quinacrine an antimalarial drug, is reported to cause phototoxicity in the skin and the eyes. The work done by Motten et al. (1999) have demonstrated a correlation between reported toxicity and generation of singlet oxygen and/or free radicals. As malaria is a disease most prevalent in regions of high light intensity, protective precautions such as clothing, sun-block, sunglasses or eye wraps should be recommended when administering antimalarial drugs (Motten *et al.*, 1999).

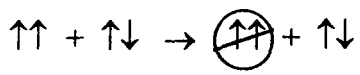
Oxygen Toxicity

A Radical Explanation

Paramagnetism and the univalent pathway

Rotating electrical charges generate magnetic fields. This applies to electrical current in a coil of wire or to a single spinning electron. The pairing of electrons with opposite spin states neutralizes this effect. Most substances are not influenced by imposed magnetic fields because the electrons they contain are all in spin-opposed pairs. Such substances are diamagnetic. O_2 is unusual in being paramagnetic. It implies unpaired electronic spins. Indeed, O_2 contains two unpaired electrons having the parallel spin state. This electronic structure constitutes a barrier to the insertion of a pair of electrons. Thus, the electrons of the incoming spin-opposed pair would be trying to join

the parallel-spinning unpaired electrons of O_2 and one of them would have the same spin state as its partner to be. This situation, schematized in reaction 1, is energetically very unfavorable, as stated by the Pauly exclusion principle:



(1)

To help this, electrons are added to O_2 one at a time. This works because electronic spins can be inverted by interaction with nuclear spins. However, this is a slow process relative to the lifetime of collisional complexes and is not likely while the reacting partners are in contact. But when the electrons are added one at a time, during separate collision event, there is time between collisions for the inversion of electronic spin. As a result, the facile route of O_2 reduction is by a series of univalent electron transfers (Fridovich, 1998).

Intermediates on the univalent pathway

The reduction of O_2 to $2H_2O$ requires four electrons. Hence, intermediates will be encountered on this univalent pathway and these are superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). It is these intermediates that are responsible for the toxicity of O_2 and defenses against that toxicity must include minimizing their production to the maximum extent possible and eliminating those whose production cannot be avoided. Most of the O_2 consumed by respiring cells is reduced by cytochrome c oxidase which, by virtue of two ferrihemes and two Cu(II) prosthetic groups, manages the four-electron reduction of O_2 to $2H_2O$ without releasing intermediates. But there are enzymes that reduce O_2 to H_2O_2, and there are both enzymic and spontaneous processes within cells that produce $O_2^{\cdot-}$. It has been estimated that approximately 0.1% of the O_2 reduced by Escherichia coli is reduced to $O_2^{\cdot-}$ (Imlay and Fridovich, 1991). Nevertheless, so great is the rate of O_2 utilization by these cells that, were the $O_2^{\cdot-}$ stable, this would have corresponded to the production of approximately 5 μmole/l intracellular $O_2^{\cdot-}$ per second. Similarly, in mitochondria, a small fraction of total O_2 reduction gives rise to $O_2^{\cdot-}$ (Gardner and Boveris, 1990).

Chemistry of free radicals

A free radical is defined as an atom or molecule that contains one or more unpaired electrons. The presence of one or more unpaired electrons causes the species to be attracted slightly to a magnetic field, and sometimes makes the species highly reactive. Such a definition embraces the atom of hydrogen (one unpaired electron) and the ions of transition metals such as iron, copper and manganese. Free radicals can be anionic, cationic or neutral. In biological and related fields, the major free radical species of interest have been those of oxygen. Radicals can easily by formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, a process known as homolytic fission. The energy required to dissociate the covalent bond can be provided by heat, electromagnetic radiation or other means (Halliwell and Gutteridge, 1985).

Molecular oxygen has two unpaired electrons in its outer orbital and its reactivity results from this biradical property. Most of the oxygen is taken up by human cells is reduced to water by the action of mitochondrial cytochrome oxidase. This requires the addition of four electrons to each oxygen molecule as seen in equation (2):

$$O_2 + 4H^{\cdot} + 4e^{\cdot} \rightarrow 2H_2O \qquad (2)$$

The intermediate steps of oxygen reduction are formation of superoxide anion radical, hydrogen peroxide and hydroxyl radical ($^{\cdot}OH$), corresponding to reduction by one, two and three electrons, respectively. Surperoxide anion radical (Table 1) contains three electrons in the orbitals (π^*, 2p) and, when dissolved in organic solvents, is extremely reactive. Two molecules of superoxide are reduced (with two protons) to $^{\cdot}OH$. At physiological pH, the low concentration of protons reduces the rate of dismutation. Step-wise single electron addition to molecular oxygen generates a unique spectrum of other reactive intermediates (Table 1). Hydrogen peroxide (H_2O_2), formed in vivo via enzymes specific for its removal, such as catalase and glutathione peroxidase, is probably more harmful than superoxide. Hydroxyl radical, produced when water is exposed to high energy ionizing radiation, is also highly reactive. $^{\cdot}OH$ are generated whenever H_2O_2 comes into contact with copper ions (Cu^{2+}) or iron ions (Fe^{2+}). Since both H_2O_2 and metal complexes are present in human, it is logical to assume that $^{\cdot}OH$ can be formed in vivo.

Table 1

Types and period of half-life of free radicals.

Intermediate	Formula	Half-life (sec.)
Hydroxyl radical	$^{\cdot}OH$	10^{-9}
Alcoxyl radical	$RO^{\cdot}$	10^{-6}
Singlet oxygen	O^{1}_{2}	10^{-5}
Peroxynitrite anion	$ONOO^{\cdot}$	0.05-1.0
Peroxyl radical	$ROO^{\cdot}$	7
Nitric oxide	$^{\cdot}NO$	1-10
Semiquinone radical	--	days
Hydrogen peroxide	H_2O_2	enzyme decomposition
Superoxide anion radical	$O_2^{\cdot -}$	enzyme decomposition

Molecular (triplet) oxygen can be electronically excited to singlet molecular oxygen, either Δ or σ–singlet oxygen. Singlet oxygen is also generated from superoxide anion radical and hydroxyl radical. In Δ –singlet oxygen, both electrons are paired with opposite spin and exist in one orbital, leaving the other orbital empty. In the σ–singlet state, the two electrons occupy different orbitals, as in the ground state, but are of opposite spins. Singlet oxygen is an especially reactive form of oxygen capable of oxidizing many molecules, including membrane lipids. Δ-singlet oxygen is more common because it is thermodynamically more stable, wherease σ–singlet oxygen rapidly converts to the Δ-state. Singlet oxygens are not truly free radicals, but are inculded in the term reactive oxygen species (ROS).

Singlet oxygen is most often generated in the laboratory by photosensitization reactions. If certain molecules are illuminated with light of a given wavelength they absorb it and the energy raises the molecule into an excited state. The excitation energy can then be transferred onto an adjacent oxygen molecule, converting it to the singlet state with the photosensitizer molecule returns to the ground state. Popular sensitizers of singlet oxygen formation in the laboratory include the dyes acridines orange, methylene blue, rose bengal and toluidine blue. Many compounds found in vivo are also effective, such as the water soluble vitamin riboflavin and its derivatives FMN. The singlet oxygen produced on illumination of these substances with light of the correct wavelength can react with other molecules present or it can attack the photosensitizer molecule itself. The chemical changes thereby produced are known as photodynamic effects. Hence illuminated solutions of flavins lose their orange color and chlorophylls their green color as they are attacked, this is called *photobleaching* (Halliwell and Gutteridge, 1985).

Reactive oxygen species can combine with other atoms or larger molecules to form alkyl- or peroxyl-radicals, e.g. in lipids (Table 1). Recent interest has centered on nitric oxide ($^{\cdot}NO$), the active moiety of endothelial derived relaxing factor, and on other nirtogen-centered free radical species, such as peroxynitrate and peroxynitrite anion. These nitrogen radicals possess long lives and high reactivity. Hemolytic cleavage of peroxynitrous acid (ONOOH) will generate the reactive radical ($^{\cdot}NO_2$), which may contribute to peroxynitrous acid toxicity.

Experimental methods for studying free radical must overcome major problems associated with their high reactivity, relatively short half-lives, and short migration distances. Free radicals can olny be studied in vitro by physico-chemical methods such as electron spin resonance (ESR). Free radicals can be measured indirectly in vivo by "trapping" them with other chemicals and then measuring the product ex vivo using ESR. Thorough assessment of free radical effects must also include measurment of natural defense systems that protect organisms from their damage, such as superoxide dismutase, catalase, glutathione peroxidase, etc. (Bergendi *et al.*, 1999).

Cellular Sources of Free radicals

Inside the cell ROS are produced enzymatically and nonenzymatically. As stated earlier, any electron-transferring protein or enzymatic system can result in the formation of ROS as "by-products" of electron transfer reactions. This "unintended" generation of ROS in mitochondria accounts for ~1-2% of total O_2 consumption under reducing conditions (Freeman and Crapo, 1982). Due to high concentrations of mitochondrial SOD, the intramitochondrial concentrations of $O_2^{\cdot-}$ are maintained at very low steady-state levels (Tyler, 1975). Thus unlike H_2O_2, which is capable of diffusing across the mitochondrial membrane into the cytoplasm (Chance *et al.*, 1979), $O_2^{\cdot-}$ generated in mitochondria is unlikely to escape into the cytoplasm. The potential for mitochondrial ROS to mediate cell signaling has gained significance in recent years, particularly the regulation of apoptosis (Banki *et al.*, 1999). There is evidence to suggest that tumor necrosis factor (TNF)-α and interleukin (IL)-1-induced apoptosis may involve mitochondria-derived ROS (Sidoti-de Fraisse et al. 1998). It has also been suggested that the mitochondria may function as an "O_2 sensor" to mediate hypoxia-induced gene transcription (Chandel *et al.*, 1998).

The endoplasmic reticulum (ER) is another membrane-bound intracellular organell. Unlike mitochondria, is primarily involved in lipid and protein biosysnthesis. Smooth ER contains enzymes that catalyze a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. The most extensively studied of these are the cytochrome P-450 and b_2 families of enzymes that can oxidize unsaturated fatty acids and xenobiotics and reduce molecular O_2 to $O_2^{\cdot-}$ and/or H_2O_2 (Capdevila *et al.*, 1981). Although there may not to be a direct link between ER-derived oxidants and growth factor signaling, there is evidence for redox regulation of ER-related functions

such as protein folding and secretion (Bader *et al.*, 1999). Bausking et al. (1991) showed that LTK, a nonreceptor tyrosine kinase (non-RTK) expressed mainly in lymphocytes, leukemia cells, and neurons, is activated by forming disulfide-linked multimers in response to thiol-oxidizing agents. It has also been suggested that an $O_2^{\cdot-}$ generating microsomal NADH oxidoreductase may function as a potential pulmonary artery O_2 sensor in pulmonary artery smooth muscle cells (Mohazzab and Wolin, 1994).

Nuclear membranes contain cytochrome oxidase and electron transport systems that resemble those of the ER but the function of which is unknown (Faulkner and Fridovich 1993). It has been postulated that electron "leaks" from these enzymatic systems may give rise to ROS that can damage cellular DNA in vivo (Halliwell and Gutteridge, 1985).

Peroxisomes are an important source of total cellular H_2O_2 production (Boveris *et al.*, 1972). They contain a number of H_2O_2-generating enzymes including glycolate oxidase, D-amino acid oxidase, urate oxidase, L-α-hydroxyl acid oxidase, and fatty acyl-CoA oxidase. Peroxisomal catalase utilizes H_2O_2 produced by these oxidases to oxidize a variety of other substrates in "peroxidative" reactions (Sidoti-do Fraisse *et al.*, 1998). These type of oxidative reaction are particularly important in liver and kidney cells in which peroxisomes detoxify a variety of toxic molecules (including ethanol) that enter the circulation. Another major function of the oxidative reactions carried out in peroxisomes is β–oxidation of fatty acids, which in mammalian cells occures in mitochondria and peroxisomes (Alberts *et al.*, 1994). Specific signaling roles have not been ascribed to peroxisome-derived oxidants, and only a small fraction generated in these intracellular organelles appears to escape peroxisomal catalase (Boveris *et al.*, 1972; Pool, 1975).

In addition to intracellular membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycle (Freeman and Crapo, 1982). The most extensively studied of these is the $O_2^{\cdot-}$ generating xanthine oxidase, which can be formed from xanthine dehydrogenase after tissue exposure to hypoxia (McKelvey *et al.*, 1988). Xanthine oxidase is widely used to generate $O_2^{\cdot-}$ in vitro to study the effect of ROS on diverse cellular processes, however, no studies have implicated a direct physiological role for endogenous xanthine oxidase in cell signaling.

Auto-oxidation of small molecules such as dopamine, epinephrine, flavins, and hydroquinones can be an important source of intracellular ROS production (Freeman and Crapo, 1982). In most cases, the direct product of such auto-oxidation reactions is $O_2^{\cdot -}$. Although there is no known role for auto-oxidation of small molecules in growth factor and/or cytokine signaling, such reactions may induce oxidative stress and alter the overall cellular redox state. There is a suggestion that the pro-oxidant effects of dopamine auto-oxidation may be involved in the dopamine-induced apoptosis that is implicated in the pathogenesis of neurodegenerative disease such as Prakinson's (Offen *et al.*, 1997).

Plasma membrane-associated oxidases have been implicated in most growth factor- and/or cytokine-stimulated oxidant production (Krieger-Brauer and Kather 1995, Sundaresan *et al.*, 1995), although the precise enzymatic sources have yet to be fully characterized. The best characterized of the plasma membrane oxidases in general is the phagocytic NADPH oxidase, which serves specialized function in host defense against invading microorganisms. This multicomponent enzyme catalyzes the one-electron reduction of O_2 to $O_2^{\cdot -}$, with NADPH as the electron donor through the trans-membrane protein cytochrome b_{558}. The transfer of electrons occur from NADPH on the inner face of the plasma membrane to O_2 on the outside. During phagocytosis, the plasma membrane is internalized and wall of the phagocytic vesicle, which was once outer membrane surface now facing the interior of the vesicle. This targets the delivery of $O_2^{\cdot -}$ and its reactive metabolites internally for localized mirobicidal activity (Babior, 1999).

Enzymes involved in phospholipid metabolism are known to exist for several decades. Membrane phospholipids, in addition to their structural role in providing membrane integrity, are substrates for the action of phospholipases (PLs) PLA_2, PLC, and PLD. Although these enzymes are important for the generation of lipid second messengers, they have generally not been associated with ROS production in nonphagocytic cells. A recent report by Touyz and Schiffrin (1999), however, suggests that ANG II-induced $O_2^{\cdot -}$ production in smooth muscle cells is dependent on the PLD pathway.

PLA_2 hydrolyzes phospholipids to generate arachidonic acid. Arachidonic acid then forms the substrate for cyclo-oxygenase- and lipoxygenase (LOX)-dependent synthesis of the four major classes of eicosanoids: prostaglandins, prostacyclins, thromboxanes, and leukotrienes. These synthetic pathways involve a series of oxidation

steps that involve a number of free radical intermediates (Freeman and Crapo, 1982). Arachidonic acid metabolism, particularly involving the LOX pathway, which leads to leukotriene synthesis, was reported to generate ROS (Baud and Ardaillou, 1986; Lim *et al.* 1983, Nakamura *et al.*, 1985; Singh *et al.*, 1981). LOX activity has also been implicated in redox-regulated signaling by ANG II (Wen *et al.*, 1997), epidermal growth factor (EGR) (Mills *et al.*, 1998), and IL-1 (Bonizzi *et al.*, 1999). There is the suggestion that LOX-derived lipid peroxidation products may be involved in the oxidative stress response to asbestos (Faux and Howden, 1997). TNF-α-induced apoptosis appears to be mediated by LOX-dependent but ROS-independent mechanism (O'Donnell *et al.*, 1995). A lipid-metabolizing enzyme in fibroblasts similar to 15-LOX is shown to generate large amounts of extracellular $O_2^{\cdot -}$ that appears to be independent of flavoenzyme activity (O'Donnel and Azzi, 1996).

Photochemical Production of Oxy Radicals

Many molecules are known photodynamic sensitizers, including various acridines, porphyrins, phenothiazines, quinones, flavins, and metal derivatives such as ruthenium bipyridyl. An activated sensitizer can react directly with other molecules to produce free radicals by removal of an electron or of hydrogen, or the sensitizer can react with singlet oxygen. Another route to activated oxygen species lies in photoreduction of the molecule in the presence of a suitable hydrogen donor, followed by reaction of the reduced molecule (either one- or two-electron) with di-oxygen. The transfer of an electron to yield superoxide radical anions.

Photoreduction of riboflavin or of flavin mononucleotide is extensively used for production of superoxide anions during the reoxidation of the dihydroflavin with donors such as methions, EDTA, or NADH to facilitate the photoreduction at 365nm. Reagents which are commonly used to detect the $O_2^{\cdot -}$ include ferricytochrome c (which is reduced), tetranitromethane (reduced), nitroblue tetrazolium (reduced), and luminol (oxidized with light emission) (Greenwald, 1987).

Possible Implication of Reactive Oxygen Species.

Hydroxyl radical

·OH is an extraordinarily powerful oxidant, which attacks most organic compounds at diffusion-limited rates (Czapski, 1984). First encountered during studies of the effect of ionizing radiation on water, it can also be produced by the reduction of H_2O_2 by metal cations such as Fe(II) or Cu(I).

$$O_2^{\cdot-} + Me(n) \leftrightarrow O_2 + Me(n-1) \quad (3)$$

$$Me(n-1) + H_2O_2 \rightarrow Me(n) + {}^{-}OH + {}^{\cdot}OH \quad (4)$$

Reaction (4) above can be broken down into a series of steps as follows:

$$Fe(II) + HOOH \leftrightarrow (Fe\text{-}OOH)^{+} + H^{+} \quad (4a)$$

$$(Fe\text{-}OOH)^{+} \leftrightarrow (FeO)^{2+} + {}^{-}OH \quad (4b)$$

$$(FeO)^{2+} + H^{+} \leftrightarrow (Fe\text{-}OH)^{3+} \quad (4c)$$

$$(Fe\text{-}OH)^{3+} \leftrightarrow Fe(III) + {}^{\cdot}OH \quad (4d)$$

The powerful oxidant produced by any of the above reactions like $(FeO)^{2+}$ or $(FeOH)^{3+}$ or ·OH are highly damaging to cell and its components. This is particularly the case since Fe(III) would not exist in free solution, but rather exist in bound formed with polyanions such nucleic acids or to phospholipid membrane. In that case, the ·OH, or $(Fe\text{-}OH)^{3+}$ or $(FeO)^{2+}$ would be produced adjacent to, and would selectively attack, those critically important targets.

Cells are rich in reductants, such as thiols and enediols, and these are able to reduce Fe(III) to Fe(II), thus obiating the need for reaction (3). None the less, $O_2^{\cdot-}$ dose collaborate with H_2O_2 in producing ·OH within cells and it worked so by oxidizing the [4Fe-4S] clusters of dehydratases, such as aconitase, causig the release of Fe(II). In this way, $O_2^{\cdot-}$ increases the availability of iron for reactions (4a)-(4d). Liochev and Fridovich have proposed this mechanism (Liochev and Fridovich, 1994) and it was subsequently experimentally verified by Keyer and co-worker (Keyer *et al.*, 1995; Keyer and Imlay, 1996).

Superoxide anion radical

O_2^{-} is more selective in its reactivity. Means that ·OH is potentially more damaging, thus, ·OH will react with something, perhaps something expendable, within a very small radius of its site of generation, whereas O_2^{-} can diffuse a considerable

distance before it encounters a suitable, and possibly critical, target. O_2^- is the conjugate base of the hydroperoxyl radical ($HOO^\cdot$) whose pKa is approximately 4.8, and spontaneous dismutations can occur as proposed by Bielski (1978):

$$^\cdot OOH + {}^\cdot OOH \leftrightarrow H_2O_2 + O_2 \quad (5)$$

$$^\cdot OOH + O_2^- + H^+ \leftrightarrow H_2O_2 + O_2 \quad (6)$$

$$O_2^- + O_2^- + 2H^+ \leftrightarrow H_2O_2 + O_2 \quad (7)$$

O_2^- is thus intrinsically unstable in protic solvents such as water but, because reaction (7) is so slow, the higher the pH the more stable it becomes. At neutral pH, it is stable enough to oxidize compounds such as polyphenols, thiols, ascorbate, catecholamines, leukoflavins, tetrahydropterins and sulfite. More importantly, it can rapidly inactivate aconitase and similar [4F-4S]-containing dehydratases.

The limited reactivity of O_2^- with a range of metabolic intermediates (Bielski and Richter, 1977) led some to argue that O_2^- was benign and that SODs were not needed for defense (Fee 1980; Sawyer and Valentine, 1981). To counter this position, the ability of O_2^- to cause the oxidation of sulfite, thiols, catechols, catecholamines, leukoflavins, and tetrahydropterins was documented (DiGuiseppi and Fridovich, 1984). Upon reaction with O_2^- several enzymes were found to be inactivated (Kono and Fridovich, 1982; Benov and Fridovich, 1999).

Of more general importance was the observation that O_2^- and H_2O_2 could collaborate in the production of the vastly reactive hydroxyl radical (Beauchamp and Fridovich, 1970). At the time it was not appreciated that metal impurities in the phosphate buffer played a critical role in this process. When that was demonstrated (McCord and Day, 1978; Halliwell, 1978), it became apparent that the process must involve the reduction of ferric chelates by O_2^-, followed by the reduction of H_2O_2 to ^-OH + $^\cdot OH$ by the ferrous chelates. Referred to as the metal-catalyzed Haber-Weiss reaction, this process was thought possible in vivo. Others correctly objected that iron, to the extent that it was "free" in cells, would be kept reduced by abundant biological reductants such as glutathione, thus obviating the need for O_2^- (Winterbourn, 1979). Yet the collaborative interaction of O_2^- and H_2O_2 in imposing oxidative damage was made apparent by the increased sensitivity of SOD-null mutants of *Escherichia coli* to H_2O_2 (Carlioz and Touati, 1986).

Defense Systems

Indigenous System of Defenses

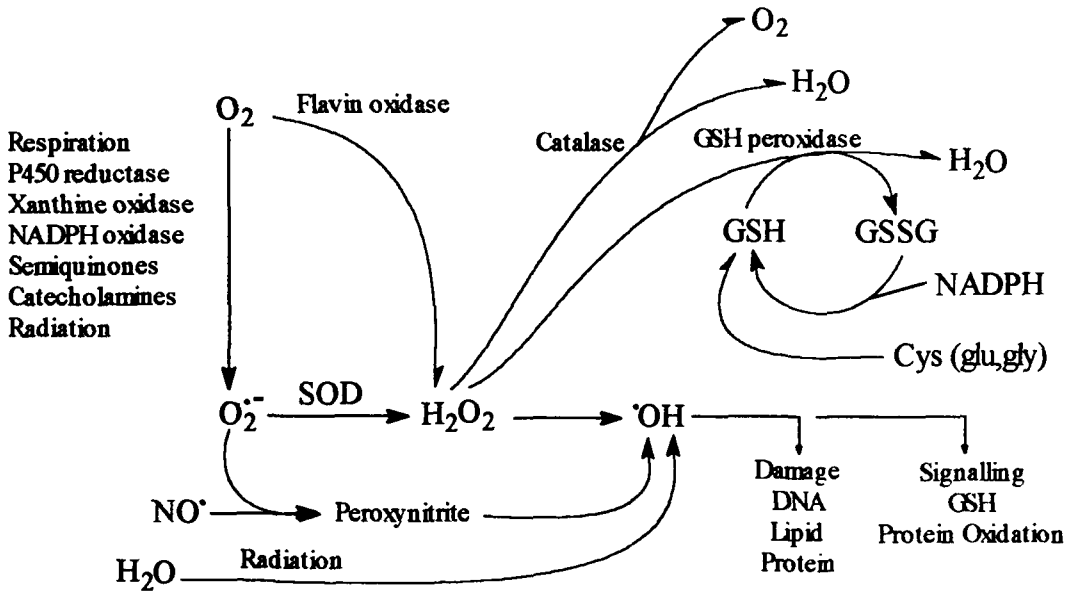
Due to both the background generation of reactive oxygen species from ambient radiation and the endogenous generation of cellular ROS outlined above, cells have evolved an elaborate series of mechanisms to minimize ROS presence and reduce damage if it occurs. Figure 2 outlines the pathways involved in ROS generation and control.

Oxidative stress was considered purely from the toxicological perspective. A relatively small number of free radicals such as the superoxide anion and the hydroxyl radical were recognized as minor by-products of oxidative phosphorylation. Britoon Chance and colleagues (1973) have determined that approximately 2% of the oxygen reduced by the mitochondrion forms $O_2^{\cdot-}$ or the dismutation product H_2O_2. This estimate has been confirmed repeatedly (Hensley *et al.*, 1998). Superoxide and peroxide react with metal ions to promote additional radical generation, with the release of the particularly reactive hydroxyl (Stadtman, 1990). Hydroxyl radicals react at nearly diffusion-limited rates with any component of the cell, including lipids, DNA, and proteins. The net result of this nonspecific free radical attack is a loss of cell integrity, enzyme function, and genomic stability (Stadtman and Berlett, 1991; Halliwell, 1999). Consequently, numerous detoxification mechanisms have evolved to deal with oxyradical stress.

Defenses against $O_2^{\cdot-}$

There are multiple defenses against $O_2^{\cdot-}$. These are the superoxide dismutases (SODs) which catalyze reaction at diffusion-limit rates. Such catalysts are abundant in aerobic cells and they keep the steady-state level of $O_2^{\cdot-}$ in the 10^{-10} $moll^{-1}$ range (Imlay and Fridovich, 1991). This would be the case if the spontaneous and the SOD-catalyzed dismutations were the only fates open to $O_2^{\cdot-}$. However, there are targets that would be

Figure 2. **Source of reactive oxygen species and mechanism of their removal from cells.**


O_2
H_2O
O_2
Flavin oxidase
Catalase
GSH peroxidase
H_2O
Respiration
P450 reductase
Xanthine oxidase
NADPH oxidase
Semiquinones
Catecholamines
Radiation
GSH
GSSG
NADPH
Cys (glu,gly)
$O_2^{\bullet-}$
SOD
H_2O_2
$^{\bullet}OH$
Damage
DNA
Lipid
Protein
Signalling
GSH
Protein Oxidation
$NO^{\bullet}$
Peroxynitrite
H_2O
Radiation

attacked by $O_2^{\cdot-}$, if they were not removed by SOD. It has recently been estimated that the SODs in *E. coli* provide approximately 95% protection for all targets susceptible to $O_2^{\cdot-}$ attack in that cell (Liochev and Fridovich, 1997). Returning to the multiplicity of SODs, we note that there are SODs that depend for their activity on active sites containing Cu and Zn, Mn, Fe and even Ni. There are SODs that are cytosolic, localized to specific subcellular organelles and also secreted from the cell. The CuZn SODs are enzymes that have Cu and Zn at their active sites. The copper undergoes valence changes during the catalytic cycle while the Zn is thought to play mainly the structural role. CuZnSODs are found in the cytosols as dimer in eukaryotic cells, in the periplasms of gram-negative bacteria, in the plastids of plants and in the extracellular spaces of mammal's (Tainer *et al.*, 1982). The Cu(II) and the Zn(II) are ligated to a bridging imidazolate that plays a role in proton conduction. Thus, upon reduction of the Cu(II) by $O_2^{\cdot-}$, the Cu-imidazolate bond is broken and the imidazolate becomes protonated. During reoxidatioin of the Cu(I) by the next $O_2^{\cdot-}$, the Cu-imidazolate bond is re-established while the protons convert the reduced $O_2^{\cdot-}$ to $HO_2^{\cdot-}$, which leaves the active site and picks up a second proton to become H_2O_2. This mechanism was proposed on kinetic grounds (Hodgson and Fridovich, 1975) and recently verified though X-ray absorption fine structure (XAFS) and crystallography (Murphy *et al.*, 1997). The MnSODs, which are as active as the CuZnSODs, but are unrelated as judged by sequence, may be dimeric or tetramic. They contain one Mn(III) per subunit and their structures have been determined by X-ray crystallography (Wagner *et al.*, 1993). The *E. coli* MnSOD is dimeric and it is not ordinarily produced when the cells are growing anaerobically (Fridovich, 1998). However, it is induced under aeration and is further induced by compounds that can increase intracellular $O_2^{\cdot-}$ production. The homotetrameric nickel-containing SOD has recently been described from *Streptomyces griseus*, which also contains a homotetrameric FeSOD.

However, SOD deficiency in the organism results in increased sensibility to hyperoxia as can be noticed in children with monosomy 21. These children may have cardiac murmur, bone abnormalities and other morphological disorders. The increased production of superoxide and hydrogen peroxide leads to, under certain conditions, DNA damage, while these reactive forms of oxygen do not interact directly with DNA

but probably by means of another highly toxic metabolite, hydroxyl radical, which can be produced from them by Fenton type reaction (Bergendi *et al.*, 1999)

Defenses against H_2O_2

The catalases and peroxidases, dismute $2H_2O_2$ into $O_2 + 2H_2O$, using various reductants, are the enzymes that deal with H_2O_2. Some of the peroxidases can also reduce alkyl hydroperoxides to the corresponding alcohols. As was the case with the SODs, the catalases and peroxidases constitute a diverse family of enzymes. Most of these are ferriheme enzymes, and their action involves the divalent oxidation of the heme to an Fe(IV) π cation radical by H_2O_2, followed by divalent reduction by H_2O_2, in the case of catalase, and by two successive univalent reductions by the organic substrate, in the case of the peroxidases (Dolphin *et al.*, 1971).

Mammalian catalase are homotetrameric ferriheme-containing enzymes whose subunit mass is approximately 60 kDa. These enzymes are most efficient when dealing with relatively high concentration of H_2O_2 because their Km for H_2O_2 lies in the millimolar range. Hence catalase is packaged into peroxisomes, along with many H_2O_2-producing enzymes. Mammalian catalase can also act as a peroxidase towards a few small molecules such as methanol, ethanol, nitrite and formate. Thus, it can use H_2O_2 to oxidize these substrates, which are small enough to gain access to the heme iron. The structure, as determined by X-ray crystallography, indicates that the heme lies deeply buried in the protein and is thus accessible only to small substrates (Reid III *et al.*, 1981). Catalase contains tightly bound NADPH, which may function to prevent the accumulation of an inactive Fe(IV) form of the enzyme (Kirkman *et al.*, 1987).

Exogenous System of Defenses

Studies of antioxidants are important because they can be used to protect polymers and foodstuffs against oxidative damage, e.g. during sterilization of food by ionizing radiation, and for possible use in the treatment of patients who ingested compounds that increase lipid peroxidation in vivo, and recently it has shown possibility of therapeutic benefit in the treatment of cancer (Brennan, 2000).

Ascorbic acid is an important antioxidant of human plasma. It is able to rapidly react with many reactive oxygen species, especially with peroxyl radical, together with the fact that ascorbic acid forms with radicals a low reactive semidehydroascorbate

radical also. Ascorbic acid is a significant antioxidant in the absence of transition metal ions, while in their presence its prooxidative properties are preferred. Recommended high doses of vitamin C (sometime even more than 10 g/day) need not be harmful for a healthy human, whereas they need not be useful in some diseases (Halliwell, 1991).

α-Tocopherol (vitamin E) itself is widely used in many foodstuffs, it is concentrated in the adrenal glands and in blood lipoproteins. It is the major, if not the only, lipid soluble antioxidant in human blood plasma. Vitamin E both quenches and reacts with singlet oxygen and could therefore protect the membrane against this species. It also reacts with the superoxide radical, although this is probably less important since the reaction is slow and $O_2^{\cdot -}$ does not initiate lipid peroxidation. In most membranes vitamin E can react with lipid peroxy radical to form vitamin E radical that are insufficiently reactive to abstract H from the membrane lipid. It thus interrupts the chain reaction of lipid peroxidation by acting as a chain terminator. The vitamin E radical produced are fairly stable because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure, so increasing stability. Furthermore, the butylated hydroxyanisole (BHA) is very often added to foodstuffs. It acts as an antioxidant by hydrogen donation, which is common to all the phenolic antioxidants. For example, addition of BHA to fat, e.g. butter, increases its storage life from a few months to a few years (Halliwell and Gutteridge, 1985).

Function of Reactive Oxygen Species

Development of Limb and Respiratory Muscle Fatigue

Many reports have indicated that reactive oxygen species represent one of the factors capable of modulating the rate of development of limb and respiratory muscle fatigue. Free radical production by contracting muscle has been demonstrated by direct, e.g. EPR and ESR studies, (Davies *et al.*, 1982; Borzone *et al.*, 1994). Indirectly lipid peroxidation (Brady *et al.*, 1979), protein carbonyl formation (Witt *et al.*, 1992, glutathione oxidation (Anzueto *et al.*, 1992) processes are the index of free radical damage.

Process After the Phagocytosis by Macrophages

Most studies on biochemistry of phagocytosis have carried out using neutrophils and macrophages, especially pulmonary macrophages since they are the only macrophages that can readily be obtained from humans. At the onset of phagocytosis, however, the cell shows a marked increase in oxygen uptake that is not prevented by cyanide, and so is unrelated to mitochondrial electron transport. This respiratory burst can be ten or twenty times the resting respiratory rate in neutrophils, but is less marked in macrophages. Some strains of bacteria are quickly killed by hydrogen peroxide, which will be formed from $O_2^{\cdot -}$ by the dismutation reaction. Also, the $^{\cdot}OH$, formed from $O_2^{\cdot -}$ and hydrogen peroxide by an iron-catalysed Haber-Weiss reaction may sometimes participate in killing (Halliwell and Gutteridge, 1985). Nitric oxide and nitrates are produced in vitro by macrophages incubated in the presence of LPS and IFN-α. They are originated from the semi essential amino acid arginine by the activity of nitric oxide synthase (Stuehr and Marletta, 1987).

Free Radical and Inflammatory Diseases

Reactive oxygen species can also damage host tissue especially in the vicinity of the inflammatory site. Reactive oxygen species have been reported to be involved in tissue injury associated with a number of inflammatory diseases including rheumatoid arthritis, atherosclerosis, adult respiratory distress syndrome, ischemia-reperfusion injury of the heart, brain, pancrease, and others (Stvrtinova *et al.*, 1995). The essential role played by the NADPH oxidase in protecting the body from infection is unequivocally demonstrated by a rare genetic disorder known as chronic granulomatous disease. Chronic granulomatous disease is characterized by genetic mutations in one of the required NADPH oxidase component proteins which result in an inactive oxidase and a shortage of hydrogen peroxide for the myeloperoxidase system (Carsky *et al.*, 1993). Patients with Chronic granulomatous disease experience severe recurrent bacterial and fungal infections that are somewhat resistant to conventional treatments (Carsky *et al.*, 1993).

Free Radicals in Cardiovascular Disease

Most cardiovascular disease events are the consequence of a thrombotic occlusion in diseased arteries containing established atherosclerotic plaques.

Pathological observations have shown that most lipid material is derived originally from circulating low-density lipoprotein (LDL) particles which are taken up by macrophages and other cells of the vascular wall. However, oxidative modification of LDL particles, which occur mainly through the effect of reactive oxygen species, seems to be a critical event in facilitating their rapid uptake into the cells of the vascular wall via an alternative scavenger receptor (Steinberg *et al.*, 1989). Oxidized LDL has other deleterious effect, it is a chemoattractant and enhances the recruitment of monocyte/macrophages into the intima, it is cytotoxic toward endothelial cells and it inactivates nitric oxide released by endothelium. These effects all contribute to the endothelial dysfunction which has been found in association with the important coronary risk factors. The endothelium normally plays a pivotal role in healthy vascular function by controlling vascular tone, cellular proliferation and haemostasis largely via the production of nitric oxide. The presence of endothelial dysfunction is thought to facilitate the subsequent development of vascular disease (Maxwell, 2000).

Reactive Oxygen Species and Neuronal Death

Neuron cell death contributes to some of the most debilitating diseases of the brain, including stroke, brain trauma, Alzheimer, Parkinson, and Huntington diseases. Even in diseases that are largely considered non-neurological, such as AIDS, there can be neuro-degeneration that due to its irreversibility, may be a lifelong consequence once the pathogenesis is halted (Zhang *et al.*, 1998).

Another active area of research and clinical investigation has been the role of reactive oxygen species in the death of neurons. Energy transport and utilization by living organisms is carried out through oxidative phosphorylation, the transfer of electrons in a series of chemical reactions within the mitochondrion. Of course, injuries that break blood vessels can expose neurons to the free-radical-forming catalyst iron. The Fenton reaction, through which iron catalyzes the production of hydroxyl radical, often is initiated experimentally by exposing neurons to ferric iron. Another clinically relevant class of compounds that can lead to increase in free-radical production within neurons is the amyloid peptides. Free-radical elevation is a shared effect of the prion protein implicated in bovine spongioform encephalopathy (mad cow disease), the Alzheimer –amylid peptide, and amylin. The peptide –amyloid is the main constituent of plaques that are the hallmarks of Alzheimer disease. It has been shown to directly

generate free radicals in aqueous solution even in the absence of cellular components (Hensley *et al.*, 1994). The stable by-products of reactive oxygen species (oxidized lipids and modified proteins) can be detected in Alzheimer disease as well as other neurodegenerative diseases (Hutchins and Barger, 1998).

Reactive Oxygen and Signal Transduction

Reactive oxygen species formed in the mitochondria and in the cytosol are important determinants of the redox state of protein cysteinyl residues, and therefore, they constitute a common regulatory mechanism of protein conformation and function. Reactive oxygen species-dependent redox cycling of cysteinyl thiols is also critical for the establishment of the protein-protein and protein-DNA interaction that determine many aspects of a signal transduction pathway (Ziegler, 1985).

Glutathione is the key regulator of the redox state of protein cysteinyl thiols. These thiols will react with GSSG if their pKa value is enough to generate a reduction potential greater than that of glutathione thiolate anion. Reactivity will tend to increase if electron-withdrawing substituents, such as basic amino acids, are in close proximity to a cysteinyl residue, because they will tend to decrease its pKa (Thomas, 1995). The intracellular level of GSSG increases from metabolism of H_2O_2 by glutathione peroxidase and decreases from the export of GSSG out of the cell and also through glutathione reductase-and NADPH-mediated reconversion of GSSG to GSH (Cotgreave *et al.*, 1988). By far, GSH is the major form of cellular glutathione, typical GSH/GSSG ratios in normal mouse liver tissue range from 50 to 200 (Jaeschke, 1990). Because of the low concentrations of GSSG relative to GSH, small increase in the oxidation of GSH to GSSG resulting from increase in reactive oxygen species and H_2O_2 metabolism will tend to produce large increase in GSSG and in steady state ratios of GSSG/GSH. GSSG increase will promote oxidation of protein cysteinyl thiols, shifting the equilibrium of thiol-disulfide exchange significantly in the direction of mixed disulfide formation and, ultimately, changing protein conformation (Thomas *et al.*, 1995).

Reactive oxygen species not only regulate the activity of pre-existing proteins, they also are responsible for inducing the expression of many genes (Keyes and Tyrrell, 1989), and for the perturbation of the signal transduction circuits responsible for maintenance of concerted patterns of gene expression (Schulze-Osthoff *et al.*, 1997). In particular, reactive oxygen species are critical in the regulation of transcription factors

in the AP-1 (Devary *et al.*, 1991). NF-kB (Toledano and Leonard, 1991), and AP-2 (Grether-Beck, 1996) the three transcription factors families that have crucial functions in proliferation, differentiation, and morphogenesis. Reactive oxygen species signaling pathway for AP-1 and NF-kB are activated in enucleated cells and in the absence of protein synthesis, indicating that DNA damage or nuclear factors are not required for their activation (Devary *et al.*, 1993).

Protein Modification Studies

There is a great deal of radiobiological literature on free radical interactions with nucleic acids and these interactions have recently been coupled to the demonstration of expected oxidation products in human urine. Hydroxyl radicals are known to fragment polysaccharides (e.g. hyaluronic acid) and free radicals participate in lipid peroxidation (Slater, 1984). In contrast, little is known of the interactions of free radicals with proteins. Proteins may thus be critical targets because they are present inside and outside the cell in high concentrations and, since many are catalytic in nature, modifications by free radicals may have an amplified effect on their activity i.e. susceptibility to proteolysis, inactivation and degradation etc.

Protein Degradation as an Index of Oxidative Stress

Reactive oxygen species are known to participate in numerous physiological and pathological processes. Situations that augment oxidant exposure, or compromise antioxidant capacity, are commonly referred to as oxidative stress. Oxidative stress can result from exogenous sources such as redox-active xenobiotics (Davies and Doroshow, 1986; Doroshow and Davies, 1986; Marcillat *et al.*, 1989) or from increased in endogenous oxidative metabolism i.e., mitochondrial electron transport (Davies *et al.*, 1982). Regardless of its source, oxidative stress has been found to effect the behavior of several different cell types.

Oxidative stress, as already mentioned, is the most ubiquitous stress to which mammalian cells are subjected (Sies, 1991). The mechanism of triggering of the stress response is under active discussion, both in general (Staus *et al.*, 1987; Morimoto *et al.*,

1990) and as a consequence of oxidative stress (Morimoto *et al.*, 1990; Tartaglia *et al.*, 1991).

The Involvement of Reactive Oxygen Species

All aerobic cells posses a number of repair and detoxification mechanisms trying to prevent the cells from oxidant-induced damage. In a normally functioning physiological system, there exits a balance between the production of oxidising species and defense against them. Cellular macromolecules, including proteins, are damaged permanently by free radicals. Oxidative damage to nucleic acids is subject to repair by highly efficient excision/insertion mechanisms, the repair of damage to proteins appears limited to the reduction of oxidized derivatives of the sulfur-containing amino acid residues. Repair of other kinds of protein oxidation has not been demonstrated. Instead, the damaged proteins are targeted for degradation to amino acid constituents by the action of various endogenous protease, including, cathepsin c, calpain, trypsin and especially the 20s proteosome (Grune *et al.*, 1996; Rivett, 1986), whose activity is also under metabolic control by diverse regulatory factors, including the concentrations of enzyme substrates, ubiquitination, and various inhibitors like crosslinked proteins (Friguet *et al.*, 1994), glycation/glycoxidation of protein conjugates (Kristal and Yu. 1992), etc.

In a complex structure like a protein, in addition to modification of amino acid side chain, oxidation reactions were able to destroy, at least partially, the secondary and tertiary structure of the macromolecule. This process is accompanied by an exposure of hydrophobic domains to the protein surface (Pacifici *et al.*, 1993). Hydrophobic surface domains of proteins tend to stick together and form non-covalent aggregates. This process is often accelerated by electrostatic interactions. In addition to the cross-linking reactions which are able to form an insoluble net of covalently cross-linked protein molecules. These crosslinking reactions may be due to the action of the oxidant itself. like the formation of the 2,2-bi-phenyl crosslink from two tyrosyl radicals, or due to the action of cross-linking sugars or lipids (Grune *et al.*, 1997). The direct reaction of several oxidants with proteins may results in protein fragments, that means, oxidants are able to cleave the polypeptide backbone chemically. The cleavage occurs at the C_α-atom of the polypeptide. Therefore, the result of an oxidative cleavage of a polypeptide are modified peptides, like in the case of degradation by proteolytic enzymes (Grune *et al.*,

1997; Stadtman, 1993). Although the formation of peptide fragments was reported in vitro, evidence do exist, that such peptide fragments are formed due to oxidative stress in living cells (Stadtman, 1993).

Susceptibility of Radical-Damaged Proteins to Enzymatic Proteolysis

A number of processes known to occur in vivo may, contribute either directly or indirectly, to the formation of damaging free radicals. Examples include the reparatory burst of phagocytes (Clark *et al.*, 1985), mitochondrial electron transport (Dean and Pollak, 1985), mitochondrial degradation during reticulocyte maturation, seems to depend on radicals produced by an endogenous lipoxygenase (Rapoport *et al.*, 1985). Furthermore, Dean and Pollak (1985) found that isolated rat liver mitochondria in state four (limited by availability of ADP) degrade their endogenously synthesized protein faster when radical fluxes are enhanced (by chain blockers and uncouplers) as compared to when they are minimized. Chloroplast 32 Kda protein is also degraded more rapidly when radical fluxes are enhanced by illumination (Matto *et al.*, 1984). In a few cases, endogenous agents which may increase radical generation are known to enhance proteolysis, for instance, phenylhydrazine increases breakdown of haemoglobin in reticulocytes (Goldberg and Boches, 1982). It has been also shown that free radicals can fragment monoamine oxidase, a protein in outer mitochondrial membrane (Dean *et al.*, 1986). Cellular proteolysis requires ATP and so changes in ATP concentration can influence proteolysis (Khairallah *et al.*, 1984). It is interesting that depletion of cellular reductants (such as NADH and Glutathione (GSH)) is often associated with enhanced proteolysis (Khairallah *et al.*, 1984). One explanation might be the decreased repair of radical damage to proteins under conditions of oxidative stress. Similarly, zinc is known to possess some antioxidant properties and there can be an inverse correlation between zinc status and proteolytic rate (Wolff and Dean, 1986). Model antioxidants, such as vitamin E, have been shown to play a critical role in retarding radical induced proteolysis, particularly that involving lipid intermediates (Dean and Cheesman, 1987).

It was demonstrated that after free radical or oxidant attack on proteins, the hydrophobicity of surface increases. Surface hydrophobicity is the key factor for the recognition and degradation of the substrate by several proteases, especially by the

proteasome (Pacifici *et al.*, 1993). With increasing radical exposure the degradation by the proteasome is increasing (Pacifici *et al.*, 1993; Grune *et al.*, 1997). Oxidized proteins tends to aggregate and form covalent cross-links, which are actually poor substrates for the proteasome, cells prevent the accumulation of oxidized proteins. It could be demonstrated that the protein turnover is increased after treatment with hydrogen peroxide or other oxidative stresses (Grune *et al.*, 1995; Grune *et al.*, 1997).

Rivett postulated first the selective degradation of oxidatively damaged glutamine synthetase by the proteasome (Rivett, 1985). It was shown that this degradation in vitro is ATP-independent, and that in fact ATP inhibits the degradation of all oxidized protein to about 10-20% (Grune *et al.*, 1997; Grune *et al.*, 1995). Nevertheless the accumulation of ubiquitinated proteins and activation of the ubiquitin system was measured after oxidative stress (Shang *et al.*, 1997). The role of the proteasomal system in the degradation of oxidized proteins in living and dividing cells was clearly demonstrated by antisense oligonucleotides against an essential proteasome subunit (Grune *et al.*, 1995). Recent studies demonstrated the function of the proteasome for the degradation of oxidized proteins in cell nucleus (Ullrich *et al.*, 1999). Therefore, it seems to be an important part of the normal function of the proteasome to degrade oxidized proteins within the cytosolic and the nuclear compartment.

Metal-Catalyzed Oxidation of Proteins

Presence of the transition metals, Fe(II) or Cu(I), and under normal conditions can catalyse the conversion of hydrogen peroxide and alkylperoxides to the highly reactive hydroxyl radical (reaction 8) or alkoxyl radical (reaction 9) which are capable of reacting with almost any organic substance.

$$H_2O_2 + Fe(II)/Cu(I) \rightarrow {}^{-}OH + {}^{\cdot}OH + Fe(III)/Cu(I) \quad (8)$$

$$ROOH\ Fe(II)/Cu(I) \rightarrow RO^{\cdot} + {}^{\cdot}OH + Fe(III)/Cu(I) \quad (9)$$

Virtually all kinds of amino acids residues of proteins are potential targets for oxidation by ·OH generated by ionizing radiation or by high concentrations of H_2O_2 and Fe(II) (Huggins *et al.*, 1993). However, since the low concentrations of iron or copper ions and H_2O_2 are present under most physiological conditions, protein damage is likely to be limited to the modification of amino acid residues at metal binding sites on the

protein, which effectively concentrate the ions. This consideration gave rise to the proposition, that the oxidation of proteins under physiological conditions is a site-specific process in which, the binding of Fe(II) or Cu(I) to metal binding sites on the protein is followed by reaction with peroxides to generate reactive species, such as ·OH and RO· radical, that will react preferentially with amino acid residues at the metal binding site (Bachur *et al.*, 1979; Levine *et al.*, 1981). Thus, the metal-binding site supports a biologically important "caged reaction". Many enzymes, especially those requiring a metal ion for activity, this will lead to loss of catalytic function (Fucci *et al.*, 1983).

Carbonyl Groups as Markers of Oxidative Damage

Reactive oxygen species-mediated oxidation of proteins leads to the conversion of histidine residues to 2-oxohistidine (Uchida and Kawakishi, 1993; Berlett *et al.*, 1996), tryptophan residues to kynurenine or N-formylkynurenine (Kikugawa *et al.*, 1994; Winchester and Lynn, 1970), tyrosine residues to dihydroxy derivatives (Huggins *et al.*, 1993; Dean *et al.*, 1993), methionine residues to methionine sulfoxide or methionine sulfone derivatives (Berlett *et al.*, 1996), leucine and valine residues to hydroxy derivatives (Garrison, 1987), and cysteine residues to disulfide derivatives (Garrison, 1987; Swallow, 1960). Of particular significance is the fact that oxidation of some amino acid residues (lysine, arginine, and proline residues) leads to the formation of carbonyl derivatives (Amici *et al.*, 1989; Creeth *et al.*, 1983; Uchida *et al.*, 1990). In addition, carbonyl derivatives of proteins are also produced as a consequence of oxidative cleavage of the peptide backbone via the –amidation pathway (Garrison, 1987; Swallow, 1960), or cleavage associated with the oxidation of glutamyl residues (Garrison, 1987; Uchida *et al.*, 1990). Carbonyl derivatives can also be formed as a consequence of secondary reactions of some amino acid side chains with lipid oxidation products, such as 4-hydroxy-2-nonenal (Friguet *et al.*, 1994; Schuenstein and Esterbauer, 1978), or with reducing sugars or their oxidation products (Mullarkey *et al.*, 1990; Creeth *et al.*, 1983). Likewise, reaction of one of the two aldehyde groups of the lipid peroxidation product, malondialadehyde, with lysine α–amino groups of proteins will yield a Schiff base possessing a carbonyl function. The interaction of reducing sugars or dicarbonyl compounds derived from the sugars can also lead to the formation of protein carbonyl derivatives. It is noteworthy that carbonyl groups of proteins

generated by one of these mechanisms may react further with the α–amino group of lysine residues in the same or another protein molecule to form intra- or inter-molecular crosslinked protein derivatives. Some of these derivatives are not only resistant to proteolytic degradation by the 20s proteosome, but may also inhibit the ability of the proteosome to degrade the oxidized forms of other proteins (Friguet *et al.*, 1994; Friguet *et al.*, 1994(B)), they may therefore, contribute to the accumulation of oxidized forms of proteins during aging and age-related diseases (Friguet *et al.*, 1994(B)).

The fact that carbonyl groups are major products of reactive oxygen species-mediated oxidation reactions have led to the development of several highly sensitive methods for the determination of protein carbonyl groups (Levine *et al.*, 1994), and the presence of carbonyl groups has become a widely accepted measure of oxidative damage under conditions of oxidative stress, aging and disease (Berlett and Stadtman, 1997). It is clear that protein carbonyl is a marker for oxidative modification of proteins. As outlined above carbonyl groups arise from a variety of oxidative processes, so that the carbonyl measurement provides a generalized or integrated assessment of oxidative damage. The absolute value of the carbonyl measurement will therefore usually be much higher than the measurement of any single product. A typical value for the carbonyl content of organs from healthy young animals is 2 nmole/mg protein (~0.10 mol carbonyl/mol protein), so that on average one of every ten protein molecules carries a carbonyl group. In contrast, a specific oxidation product may be present at very low levels. For example, dopa can be formed from the oxidation of tyrosine, and it also reacts with carbonyl reagents. In low density lipoprotein, there is approximately 1 dopa per 1,600 tyrosine residues (Dean *et al.*, 1997). Thus, it is not surprising that measurement of dopa in tissue protein would give values which are orders of magnitude less than the protein-associated carbonyl in the same sample (Dean *et al.*, 1997). Also, as outlined in this section, the oxidative modification giving rise to the carbonyl group is emerging as more than a marker of oxidative stress. The oxidative modification may be the key to dysfunction induced by oxidative stress.

Antioxidant Activity of Methionine Residues of Proteins

Methionine residues of proteins are particularly sensitive to oxidation by virtually all kinds of reactive oxygen species. Ozone (Mudd *et al.*, 1969), hydrogen peroxide (Kido and Kassell, 1975), alkyl peroxides (Chao *et al.*, 1997), peroxynitrite

(Pryor *et al.*, 1994), hypochlorous acid (Moreno and Pryor, 1992), metal catalyzed reactions (Schoneich *et al.*, 1993), ultraviolet light (Schoneich and Yang, 1996), and ionizing radiations (Garrison, 1987; Swallow, 1960), have all been shown to convert methionine residues of protein to methionine sulfoxide derivatives. But unlike oxidation of non-sulfur-containing amino acids, the oxidation of methionine residues by reactive oxygen species can be reversed. The conversion of methionine sulfoxide residues back methionine residues is catalyzed by the thioreddoxin TR(SH)2-dependent peptide methionine sulfoxide reductase, which is widely distributed in most animal tissues and in bacteria. It follows that when coupled with the NADPH-dependent reduction of oxidized thioreddoxin [TR(S-S)] by thioreddoxin reductase [TxR], the cyclic oxidation and reduction of methionine residues constitutes an antioxidant system with which almost all forms of reactive oxygen species can be converted to inactive derivative (Moskovitz *et al.*, 1998).

Interaction between intermediates in Lipid Peroxidation and Protein Degradation

Food technology studies have shown that peroxidizing lipid damages proteins. Most emphasis has been placed on protein cross-linking but there is also evidence of fragmentation (Hunt *et al.*, 1988; Wolff and Dean, 1987). These reactions with proteins may involve both, the radicals (Bedwell *et al.*, 1989) and the aldehyde generated (Hunt *et al.*, 1988) during lipid peroxidation.

Perhaps the most widely studied biological lipid/protein system is that of low density liporprotein (LDL). During peroxidation of the lipid component, the apoprotein of LDL becomes fragmented (Bedwell *et al.*, 1989; Parsharsarathy *et al.*, 1985) and there is crosslinking and residue modifications. Many studies have shown that cell-mediated alterations, in which LDL is incubated with endothelial cells, smooth muscle cells or mononuclear phagocytes from a number of species, lead to the generation of modified form(s) of LDL which are more rapidly endocytosed by macrophages (Heinecke *et al.*, 1984; Hinsbergh *et al.*, 1986).

There is much evidence of inactivation of membrane enzymes during lipid peroxidation (Dean *et al.*, 1986), because the mitochondrion is an important source of cellular superoxide anion and hydrogen peroxide. Wolff and Dean (1986) have studied interactions between mitochondrial proteins and free radicals derived either from

electron transport or from lipid peroxidation. Mitochondrial membrane monoamine oxidase was used as a model in these studies. Fragmentation was produced by $^{\cdot}OH/O_2$ and $^{\cdot}OOH$. In addition, much lipid peroxidation has been observed during radical attack (Dean, 1986).

Proflavine Action on Proteins

Serine proteases have attracted a great deal of interest as drug targets due to their widespread involvement in biological processes. Proflavine has been an extremely effective probe for elucidating the mechanism of action of these enzymes. The binding of proflavine to the serine proteinases, especially trypsin, thrombin and chymotrypsin, was accompanied by a substantial shift in the visible absorption spectrum. This spectral shift has been utilized by many investigators as a means of detecting complex formation and dissociation between proteases, proflavine, and substrates or inhibitors (Bernhard *et al.*, 1966; Fink, 1974; Koehler and Magnusson, 1974). Proflavine competitively inhibits the activity of several proteolytic enzymes. In thrombin, proflavine binds to a single specific site with micromolar affinity competing both with the charged inhibitor *p*–aminobenzamidine and with the neutral indole and related compounds. The binding of benzamidine to the specificity pocket of thrombin and the displacement of proflavine by indole lead to the idea that the acridine moiety binds adjacent to the catalytic center in a polar region of the active site. The crystallographic studies of the proflavine binding to the active site of human α–thrombin have suggested that proflavine molecule binds in the S1 pocket of the enzyme with one of the proflavine amino groups hydrogen bonded to the carboxylate of Asp-189 and with the protonated ring nitrogen which is hydrogen bonded to the carbonyl of Gly-219. Such studies indicate that the proflavine displacement assay can be used for monitoring the binding of other low molecular weight inhibitors to the S1 pocket of α–thrombin (Coni *et al.*, 1998). Moreover, thermodynamically, this binding is characterized by a change in the standard heat capacity change (ΔC_p) which proposed that a burial of a large surface area of non-polar residues brought about the observed heat capacity change (Cristofaro and Landolfi, 1994), a recent crystal sturcture of proflavine and thrombin have shown that the sodium atom identified in an extended solvent channel beneath the S1 pocket may play a role in binding of these ligands (Nienaber *et al.*, 2000). Interestingly, investigations for the action of proflavine with thiol proteases, papain and ficin, have given rise to apparent

enhancement in the catalytic activity of these two enzymes toward certain synthetic ester substrates. It was acting as a noncompetitive activator of papain by inducing a decrease in Km for the enzyme hydrolysis of its substrate (Hall and Anderson, 1974; Skalski *et al.*, 1973).

Differential action of proflavine on serine and thiol proteases have attracted Brantner and his co-worker to develop a biotechniqal method based on proflavine for the separation of proteolytic enzymes. Their procedure was applicable for purifying serine proteinases from natural materials or for removing serine proteinase contaminants from enzyme preparations, and all this was performed by preparing an affinity chromatography utilizing Sepharose aminocaproyl proflavine resin, so the enzymes which are inhibited by proflavine (serine proteinases) are tightly bound to the proflavine column and the enzymes which are activated by proflavine (The thiol proteases ficin and papain) are slightly retarded by the column, and finally those enzymes that are unaffected by proflavine are not bound. The column that they have prepared is stable, and has been used repeatedly for nine months giving the same results (Brantner *et al.*, 1976).

Diseases Related to Protein Oxidation

Elevated levels of protein carbonyls are also associated with of a number of age-related diseases, often correlating well with the progression of the disease. An increase in the carbonyl content of protein is associated with Alzheimer's disease (Hensley *et al.*, 1994), Parkinson's disease (Alam *et al.*, 1997; Floor and Wetzel, 1998), diabetes (Baynes and Thorpe, 1999), rheumatiod arthroitis (Chapman *et al.*, 1989), muscular dystrophy (Murphy and Kehrer, *et al.*, 1989), cataractogenesis (Garland *et al.*, 1988), induction of renal tumors (Uchida *et al.*, 1995), bronchopulmonary dysplasia (Gladstone *et al.*, 1994), amyloidosis (Kong *et al.*, 1996), chronic ethanol ingestion (Grattaglianno *et al.*, 1996), acute carbon tetrachloride toxicity (Sundari and Ramakrishna, 1997), amyotrophic lateral sclerosis (Bowling *et al.*, 1993), and the progerias (Oliver *et al.*, 1987). In some of these diseases, more than one kind of oxidative modification has been demonstrated. For example, Alzheimer's disease has been shown to be associated with an increase in protein carbonyl content (Smith *et al.*, 1991), in advanced glycation end products (Vitek *et al.*, 1994), in protein HNH adducts (Montine *et al.*, 1998), in nitrated tyrosine derivatives (Good *et al.*, 1996), and in redox active iron (Smith *et al.*, 1997).

Cataractogenesis. is associated with an increase in protein carbonyl group (Garland *et al.*, 1988) and methionine sulfoxide (Lund *et al.*, 1996), Parkinson's disease is associated with an increase in both protein carbonyl groups (Floor and Wetzel, 1998; Kong *et al.*, 1996) and HNH adducts (Yoritake *et al.*, 1996). It is noteworthy that in the available studies of Parkinson's disease, all patients had been treated with dopa, which is known to provoke protein oxidation (Kong *et al.*, 1996; Lavoie and Hastings, 1999). Thus, in Parkinson's disease the observed protein oxidation may be a consequence of the underlying pathophysiology or it may be a result of drug treatment.

Experimental

Materials

Chemicals used for the present study were obtained from the following sources as details below. Glass distilled water was used in all the experiments.

Chemicals	***Sources***
Acetic acid	Qualigens, India.
Acrylamide	Sisco Res. Lab. (SRL), Bombay, India.
Ammonium persulphate	Sisco Res. Lab. (SRL), Bombay, India.
Bathocuproine disulphate	Aldrich Chemical Co., U.S.A.
Bovine Serum Albumin	Sigma Chemical Co., U.S.A.
Catalase *(from bovine liver)*	Sigma Chemical Co., U.S.A.
Citric acid	B.D.H., India.
Cuprir chloride	B.D.H., India.
Deoxyribonucleic acid *(Calf Thymus Type I)*	Sigma Chemical Co., U.S.A.
Di-sodium hydrogen phosphate (Dibasic)	Qualigens, India.
Ethylenediaminetetra acetic acid	B.D.H., India.
Folins ciocalteu's phenol reagent	Sisco Res. Lab. (SRL), Bombay, India.
Glucose	Qualigens, India.
Glycerol isopropanol	Qualigens, India.
Glycine	Qualigens, India.
Hydrochloric acid	B.D.H., India.
Hydrogen sodium phosphate	Qualigens, India.
Invertase	Sigma Chemical Co., U.S.A.
Lysozyme	Sigma Chemical Co., U.S.A.
Mannitol	E. Merck, Germany.
β-mercaptoethanol	E. Merck, Germany.
N,N`-methylene bisacrylamide	Sisco Res. Lab. (SRL), Bombay, India.
Nitroblue tetrazolium	Sisco Res. Lab. (SRL), Bombay, India.
Potassim iodide	E Merck, Germany.
Proflavine *(hemisulphate)*	Sigma Chemical Co., U.S.A.
Ribonucleic acid (RNA)	Sisco Res. Lab. (SRL), Bombay, India.
Sodium azide	E. Merck, India.

Sodium Benzoate	E. Merck, India.
Sodium bicarbonate	Qualigens, India.
Sodium carbonate	Qualigens, India.
Sodium citrate	Sisco Res. Lab. (SRL), Bombay, India.
Sodium dodecyl sulphate	Sisco Res. Lab. (SRL), Bombay, India.
Sodium hydroxide	B.D.H., India.
Sodium potassium tartarate	Qualigens, India.
Sucrose	Sisco Res. Lab. (SRL), Bombay, India.
Sulphuric acid	Qualigens, India.
Superoxide dismutase *(from bovine erythrocytes)*	Sigma Chemical Co., U.S.A.
N,N,N',N'-tetramethylene diamine	Koch-light Lab. Ltd., England.
Thiourea	E. Merck, India.
Trichloroacetic acid	Glaxo, India.
Tris (hydroxy methyl) amino methane HCl	Sisco Res. Lab. (SRL), Bombay, India.
Triton X-100	B.D.H., India.
Trypsin	Sisco Res. Lab. (SRL), Bombay, India.

All other chemicals were commercial products of analytical grade.

Methods

Proflavine was dissolved in distilled water as 1 mM stock solutions just prior to experimentation and was warmed for few second to facilitate its dissociation in the stock solution. Upon addition to reaction mixtures, in the presence of buffers and at the concentrations used, proflavine remained in solution. The volumes of stock solutions added did not lead to any appreciable change in the pH of the reaction mixtures. All the solutions were prepared in the dark to prevent photoisomerization of proflavine.

Spectroscopy

The absorption spectra were obtained by using Beckman DU-40 spectrophotometer. The fluorescence spectra were recorded on a Shimadzu spectrofluorometer (Tokyo, Japan) equipped with a calculator and a plotter. In case of tryptophan fluorescence quenching, BSA (0.7 μM) was excited (λ_{max}) at 280 nm, invertase and lysozyme (0.7 μM each) were both excited at 280 nm. Spectra were read between 300-400 nm for all cases, emission slit was 10 nm for all. Appropriate controls containing the native untreated protein were run and corrections made wherever necessary. In some experiments free radical scavengers like sodium azide, superoxide dismutase (from Bovine erythrocytes), catalase (from Bovine liver), potassium iodide, sodium benzoate, sodium formate, thiourea and mannitol were included. All spectrophotometric and fluorometric experiments were done at pH 7.4 and at ambient temperature.

Detection of Superoxide Anion ($O_2^{\cdot-}$)

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayma et al. (1983). A typical assay mixture contained 50 mM potassium phosphate buffer pH 7.8, 0.033 mM NBT, 0.1 mM EDTA and 0.06% triton X-100 in a total volume of 3.0 ml. The reaction was started by the addition of the proflavine. After mixing, absorbance was recorded at 560 nm against a blank, which did not contain the compound, at different time intervals. The reaction was carried near the spectophotomer instrument to give very short time duration in the process of recording the reading. To confirm the formation of superoxide anion, superoxide dismutase (SOD)

was added to the solution before addition of the compound. Experiments were carried out in triplicate and mean values are reported.

Detection of Hydroxyl Radicals ($^{\cdot}OH$)

Treatment of deoxyribose with hydroxyl radical in phosphate buffer in the presence of oxygen cause its degradation to yield products which react on heating with thiobarbituric acid under acid conditions to form colored products as described by Halliwell and Gutteridge (1981). The reaction was carried with some modification and it was containing 10 mM sodium phosphate buffer pH 7.4, 2 mM deoxyribose and varying concentration of proflavine in a total volume of 0.5 ml. The reaction was incubated at 37°C for five hours. Then 0.5 ml of 1% (w/v) thiobarbituric acid (dissolved in 50 mM NaOH)) plus 0.5 ml 2.8% (w/v) trichloroacetic acid were added and the reaction mixture was heated at 100°C for 30 minutes and cooled after that. The absorbances at 532 nm were recorded against a blank, which did not contain the proflavine. Known scavengers of hydroxyl radical such as mannitol, thiourea and sodium formate are usually used to show its ability in preventing the formation of chromogen from deoxyribose in the given system. Experiments were carried out in triplicate and mean values are reported.

DNA Molecular Study

Preparation of Denatured DNA

Denatured DNA was prepared by heating highly polymerized calf thymus DNA solution (2mg/ml) in TNE (10 mM Tris-HCl, 10 mM NaCl, 0.2 mM EDTA, pH 7.5) at 100°C for six minutes and rapidly cooling in ice (Hadi and Goldthwait, 1971).

Alkylation of DNA and preparation of Depurinated DNA from alkylated DNA

To 20 ml solution of DNA (2mg/ml) in TNE (10 mM Tris-HCl, 10 mM NaCl, 0.2 mM EDTA, pH 7.5) 0.045 ml of 100% dimethyl sulfate was added (specific gravity 1.33) to give a DNA nucleotide to dimethyl sulfate molar ratio of 1:4. The mixture was shaken gently for one hour at room temperature. The pH was checked after every five

minutes and any decrease in pH was corrected by addition of small volumes of concentrated NaOH. The alkylated DNA was divided into two halves. The first half was dialysed overnight against 50 volumes of TNE at 4 °C. Depurinated DNA was obtained by incubating the other half at 50 °C for six hours to achieve the release of labile alkylated bases (Verly et al., 1973). The dimethyl sulfate and released alkylated bases were removed by overnight dialysis against 50 volumes of TNE at 4 °C.

Preparation of Cross-Linked DNA

Cross-linked DNA was prepared as described by Verly and Lacroix (1975). To 10 ml DNA (2mg/ml) in TNE was added 10 ml of sodium nitrite (2 M) in 0.5 M sodium acetate buffer, pH 4.5. The solution was kept at room temperature for 90 minutes and then chilled in ice. 5 ml of Na_2HPO_4 (2 M) were added to bring the pH back to 6.5. The cross-linked DNA thus formed was extensively dialysed against three changes (500 ml each) of TNE (10 mM Tris-HCl, 10 mM NaCl, 0.2 mM EDTA, pH 7.5).

Reaction of proflavine and Cu(II) with protein and tryptic digestion

The reaction mixture, in a final volume of 1 ml contained 10 mM sodium phosphate buffer, pH 7.4, 2 mg of Bovine Serum Albumin (BSA) and varying concentration of proflavine with or without 200 μM cupric chloride. After illumination with fluorescent light, the samples were further incubated for 30 minutes with 10 μg trypsin at 37 °C. The reaction was terminated with 0.1 ml of 10 mM EDTA and 0.5 ml of 10% trichloroacetic acid (TCA). The TCA soluble material was assayed by the method of Lowry et al. (1951) and the colour developed was read at 660 nm against reagent blank.

Slab Gel Electrophoresis

The reaction mixture consisted of protein (1mg/ml) and proflavine or proflavine and 200μM Cu(II) in a total volume of 1 ml. After incubation with fluorescent light at room temperature for different time intervals, the reaction was terminated with 0.25 ml of SDS-sample dye that contain 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue. The samples were

incubated at 37 °C for 45 minutes, or boiled for five minutes before loading. 10 µg of BSA was applied in each lane. The samples were separated on 10% (w/v) SDS-ploy acrylamide gels essentially performed by the method of Laemmli (1970). In case of invertase 80 µg protein was applied in each lane and the samples were separated on a gradient (5-12.5% (w/v)) polyacrylamide gels. Whereas in case of trypsin 10 µg protein was applied in each lane and the samples were separated on 15% (w/v) SDS-polyacrylamide gels, moreover, the trypsin was boiled for 3 minutes before running the reaction so we can avoid the autoproteolysis of the enzyme. Electrophoresis was performed at 100V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192 mM glycine and 25 mM Tris-HCl pH 8.8, the SDS was not added in case of native PAGE. In some experiments, 50 mM of free radical scavengers like sodium azide, potassium iodide, sodium benzoate, sodium formate, thiourea and mannitol were included in the reaction mixture of protein with proflavine as indicated.

After electrophoresis, the gels were stained with silver nitrate staining method, which can detect proteins within 30 minutes as described recently by Nesterenko et al. (1994) at room temperature.

Enzyme Assays

Assay of Invertase

The activity of β–D-Fructofuranosidase [EC 3.2.1.26], more commonly known as invertase, was estimated as described by Bernfeld (1955). The reaction mixture contained in a total volume of 0.3 ml. 150 µl of 0.2 M sodium acetate buffer pH 4.8, 100 µl of suitably diluted enzyme solution and 50 µl of 0.5 M sucrose solution. The assay mixture was incubated at 37 °C for 10 minutes and the reaction stopped by addition of 0.2 ml of 0.5 M sodium phosphate buffer pH 7.0 which markedly slowed down the reaction and rendered the enzyme more sensitive to heat treatment which is followed by incubation at 100 °C for 3 minutes. Glucose thus released was determined by adding 1.0 ml of dinitrosalicylic acid (DNS) reagent to the reaction mixture and the tubes were incubated at room temperature for 5 minutes. After heating in boiling water bath for 5 minutes the tubes were cooled and 3 ml distilled water was added to the reaction mixture. The intensity of colour was read at 540 nm. One unit of invertase is

the amount that hydrolyses 1.0 μM of sucrose to glucose and fructose per 10 minutes at 37°C.

Assay of Trypsin

Trypsin [EC 3.4.21.4] was assayed by the method of Schroeder et al. (1968). The reaction mixture in a total volume of 2 ml contained 15 μg of trypsin in 10 mM sodium phosphate buffer pH 7.4 and bovine serum albumin as a substrate with a concentration of 1mg/ml in the reaction mixture. The reaction was incubated at 37 °C and started by the addition of trypsin. After incubating for 30 minutes, the reaction was terminated by the addition of 0.1 ml of 10 mM EDTA and 0.5 ml of 10% trichloroacetic acid (TCA). The samples were then centrifuged at 2500 rpm for 10 minutes to remove the undigested protein (precipitate) and the TCA soluble material in the supernatant was used for determining the acid soluble peptide using the method of Lowry et al. (1951). The appropriate aliquots of supernatant was taken in a set of tubes and final volume was made up to 1 ml with 0.01 M sodium phosphate buffer pH 8.0. Five ml of alkaline copper reagent (containing one part of 0.5% (w/v) copper sulphate in 1% (w/v) sodium potassium tartarate and 50 parts of 2% (w/v) sodium hydroxide) was added, incubated for 10 minutes at room temperature. 0.5 ml of 1.0 N Folin-Ciocalteu's phenol reagent was added and vortexed. The colour developed was read at 660 nm after 30 minutes against a reagent blank. A standard curve was prepared using BSA as standard. Reading was monitored at 280 nm in Bechman DU-40.

Results

Generation of superoxide anion ($O_2^{\cdot-}$) by proflavine upon illumination with fluorescent light

It is well established by the studies of Piette and co-workers (Peitte *et al.*, 1978; Piette *et al.*, 1979; Piette *et al.*, 1981; Piette *et al.*, 1982) that illumination of proflavine with visible light in presence of macromolecules such as DNA results in the generation of free radicals under aerobic conditions. We have shown that proflavine alone can generate superoxide anion in visible light (Figure 3-A). The increase in absorption at 560 nm upon the reduction of nitroblue tetrazolium (NBT) to formazan as described by Nakayama et al. (1983) is taken as the index for the generation of $O_2^{\cdot-}$. From the figure it is also evident that the production of superoxide anion is concentration and time dependent (Figure 3 (A, B)). The production of superoxide anion increased linearly up to four hours of illumination and then leveled off showing no further increase. Presence of 100 μg/ml SOD in the NBT reaction gave 55% inhibition, indicating that $O_2^{\cdot-}$ is one of the reactive oxygen species generated by photoilluminated proflavine. Presence of transition metal ions such as Cu(II) or Fe(III) did not alter the production of superoxide by the photoilluminated proflavine (Figure 4).

Generation of hydroxyl radical ($^{\cdot}OH$) by proflavine upon illumination with fluorescent light

Proflavine is shown to generate $O_2^{\cdot-}$. As the presence of SOD resulted in 55% inhibition only. The possible involvement of other reactive oxygen species such as hydroxyl radical ($^{\cdot}OH$) was tested especially in the presence of metal ion such as Cu(II) as it is known to participate in Fenton like reaction.

The generation of hydroxyl radical by photoilluminated proflavine with or without Cu(II) was monitored as described in Figure 5. The assay is based on the fact that degradation of deoxyribose by free radicals will yield products that react with thiobarbituric acid upon heating under acid conditions to form colored product, readable at 532 nm (Halliwell and Gutteridge, 1981). Increasing concentration of proflavine led to increased production of TBA reactive material. At a fixed concentration of proflavine an increasing copper concentrations resulted in a dose dependent increase in the production of TBA reactive material (Figure 6). Formation of colored compound from

Figure 3. Photogeneration of superoxide anion ($O_2^{\cdot-}$) by proflavine.

(A) Effect of increasing concentration of proflavine on the formation of superoxide anion ($O_2^{\cdot-}$) as measured by the reduction of NBT to formazan after incubation at 25°C for five hours in fluorescent light. See 'Methods' for details.

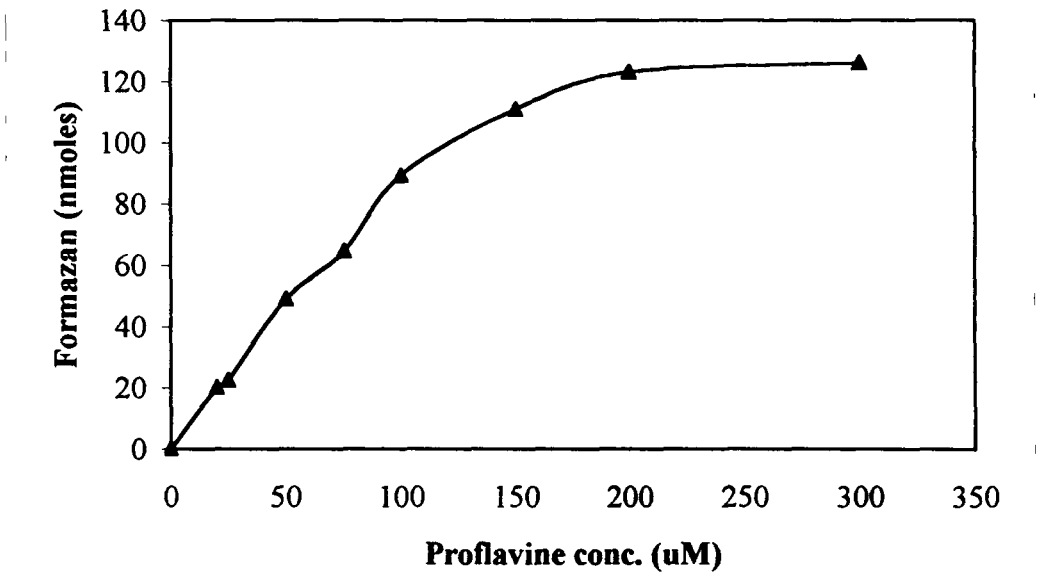
(B) Inhibition of proflavine mediated formation of superoxide anion by superoxide dismutase (SOD). The 3.0 ml reaction mixture contained 200μM proflavine, were incubated for different time intervals at 25°C.

(-▲-) Proflavine alone.

(-Δ-) Proflavine in presence of 100 μg SOD.

(-❑-) Proflavine alone in dark.

A


Formazan (nmoles)
140
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Proflavine conc. (uM)

B

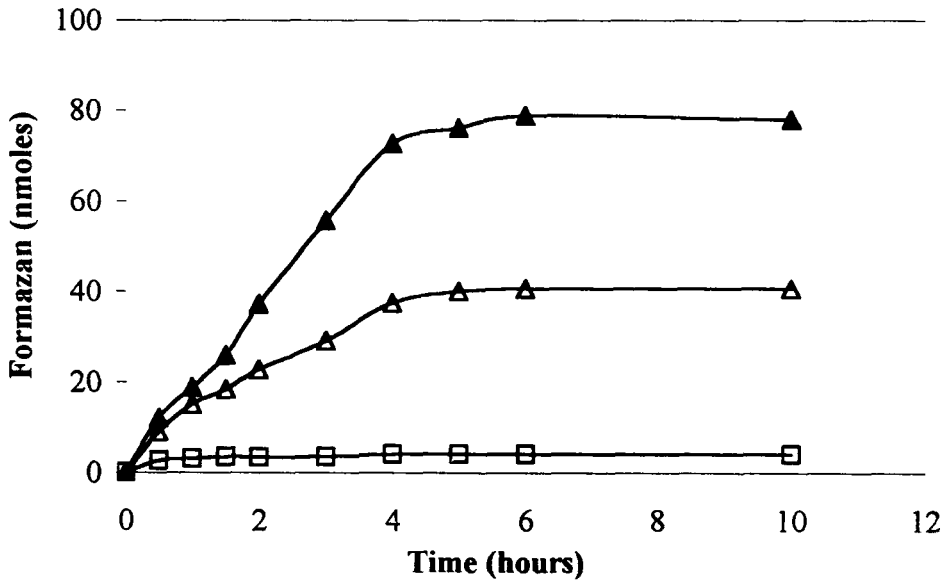

Formazan (nmoles)
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (hours)

Figure 4. Effect of transition metal ions Cu(II) & Fe(III) on superoxide anion ($O_2^{\cdot-}$) formation by proflavine.

100 μM of Cu(II) or Fe(III) were added to the reaction mixture that contained 200 μM proflavine and the reduction of NBT to formazan was recorded at 560 nm.

(-▲-) Proflavine alone.

(-■-) Proflavine + Cu(II).

(-●-) Proflavine + Fe(III).

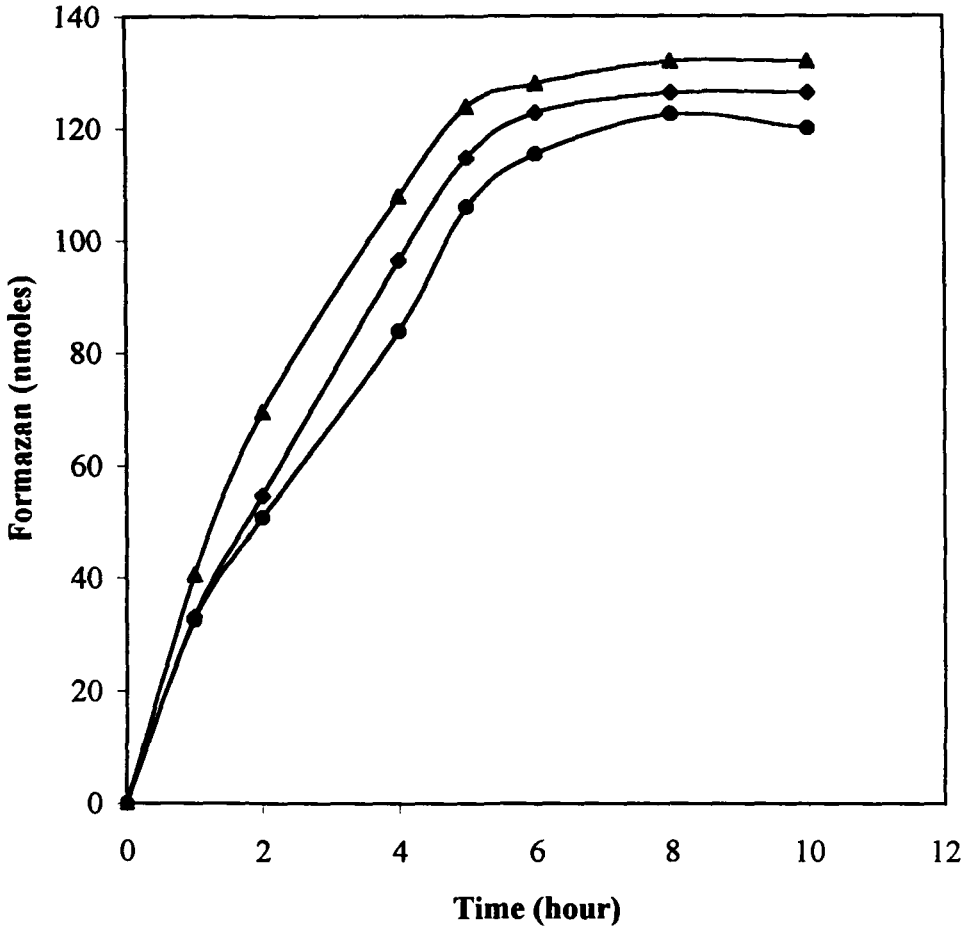

140
120
100
80
60
40
20
0
Formazan (nmoles)
0
2
4
6
8
10
12
Time (hour)

Figure 5. Photogeneration of hydroxyl radical (·OH) by proflavine.

The reaction mixture in a total volume of 0.5 ml contained 10 mM sodium phosphate buffer pH 7.4, 2 mM deoxyribose and varying concentration of proflavine with or without 100 μM Cu(II). All reactions were incubated in fluorescent light for five hours. Details are described in 'Methods'.

(-▲-) proflavine alone.

(-●-) proflavine and Cu(II).

(-Δ-) proflavine alone in dark.

(-○-) proflavine and Cu(II) in dark.

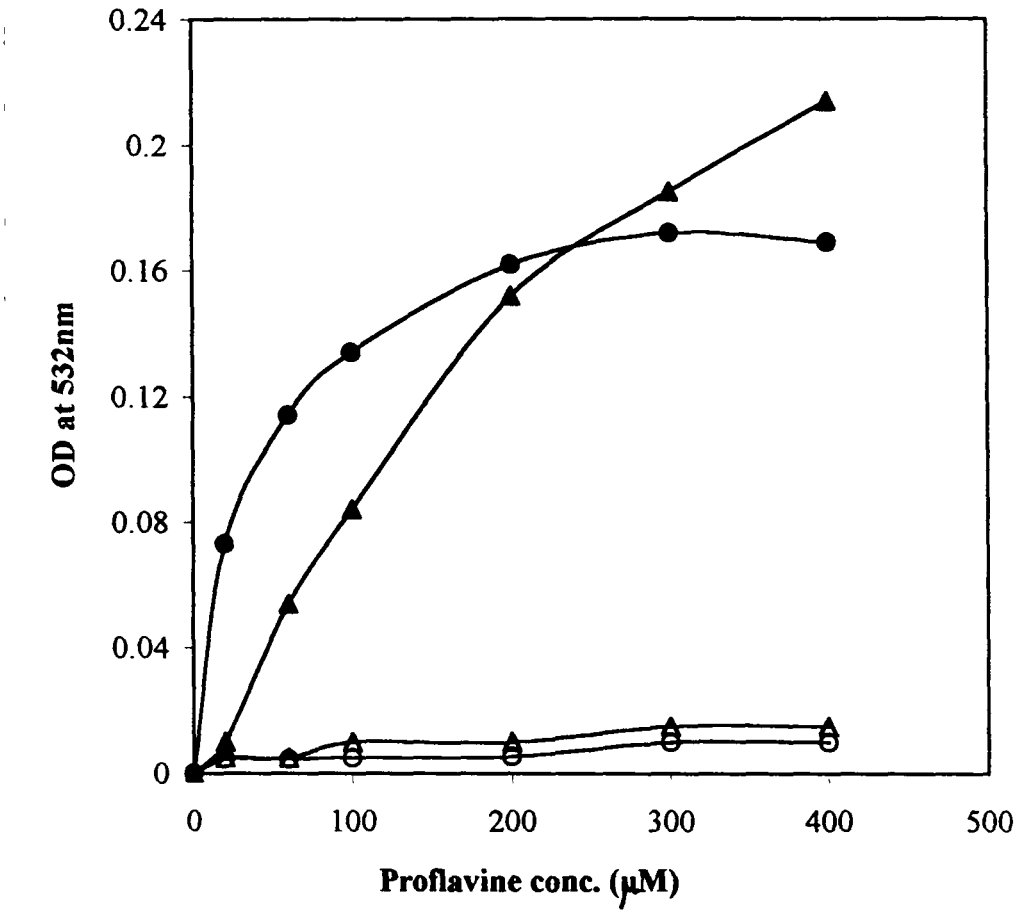
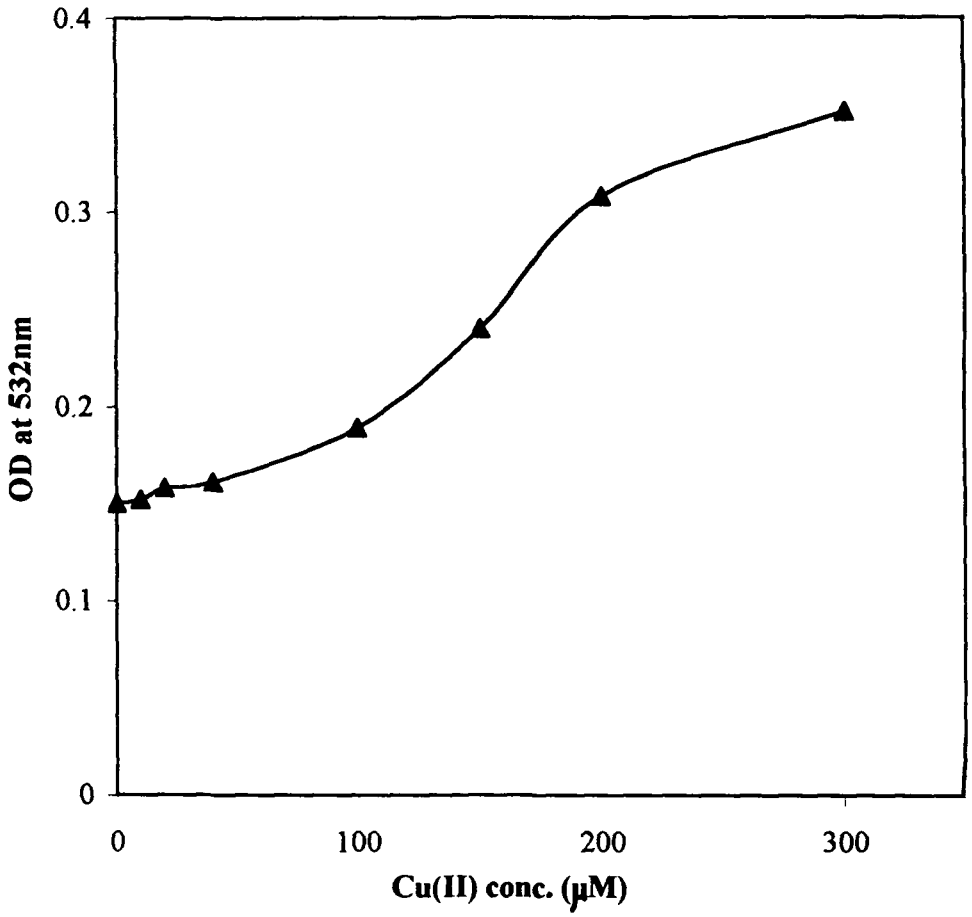

0.24
0.2
0.16
0.12
0.08
0.04
0
OD at 532nm
0
100
200
300
400
500
Proflavine conc. (μM)

Figure 6. Effect of increasing concentration of Cu(II) on the photogeneration of hydroxyl radical by proflavine.

The reaction mixture in a volume of 0.5 ml contained 10 mM sodium phosphate buffer pH 7.4, 2 mM deoxyribose and 200 μM proflavine with varying concentration of Cu(II). Reaction mixture was incubated in fluorescent light at 25°C for five hours. See 'Methods' for more details.


0.4
0.3
0.2
0.1
0
OD at 532nm
0
100
200
300
Cu(II) conc. (μM)

deoxyribose is not very specific since it is likely that other oxidizing species can degrade deoxyribose, it was therefore, necessary to use scavengers of the hydroxyl radical in the above reaction. The degradation of deoxyribose when carried out in presence of various hydroxyl radical scavengers like thiourea, sodium formate and mannitol, maximum inhibition of 46% was given by sodium formate followed by mannitol and very little with thiourea (Figure 7). The inhibition was more significant when Cu(II) was present with proflavine in the reaction mixture.

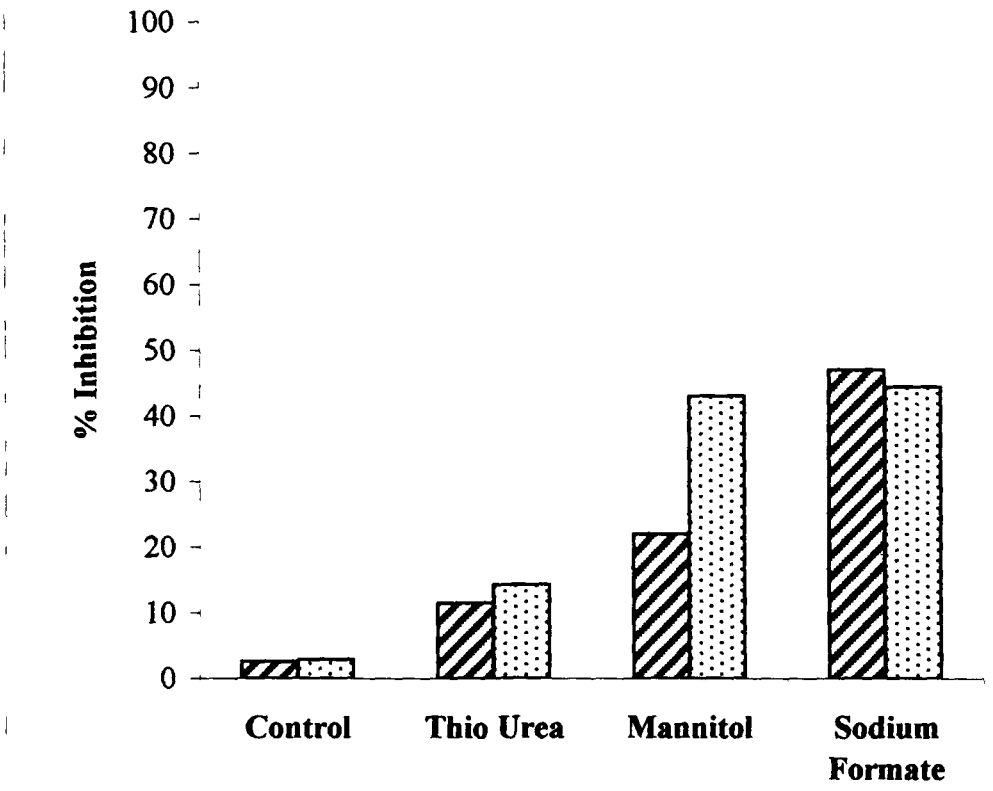
Spectral studies of proflavine

Acridines are a well-known class of heterocyclic aromatic photosensitizers. Proflavine a derivative of acridine, in aqueous solution exhibits strong absorption maxima with a peak of absorbance at 443 nm and another at 260 nm as described by Marini et al. (1971). We have determined the spectral changes in proflavine as a function of time of illumination with visible light (Figure 8). Upon incubation in fluorescent light for four hours both the peaks decreased significantly suggesting photodegradation of proflavine. Various free radical scavengers such as sodium azide, potassium iodide, thiourea, mannitol, sodium formate, superoxide dismutase and catalase when present during illumination, photodegradation of proflavine was significantly inhibited. Potassium iodide, thiourea and sodium azide which are specific quenchers of triplet oxygen (O^3_2), ·OH and singlet oxygen (O^1_2) respectively, gave around 75%, 53% and 27% inhibition respectively, suggesting O^3_2 and O^1_2 as a major reactive oxygen species generated upon photoillumination of proflavine in addition to ·OH (Figure 9).

Cu(II) when present in the reaction mixture together with proflavine have partially restored both the peaks (Figure 10).While presence of protein did not help or prevent photodegradation of proflavine (Figure 11). Furthermore, presence of double stranded DNA, single stranded DNA and RNA all caused a shift in 443nm peak of proflavine which is indicative of binding with the double stranded DNA, denatured DNA and RNA. However, the shift was maximum with double stranded DNA (Figure 12-A). Moreover, after four hour of incubation in fluorescent light the double stranded DNA was able to inhibit photodegradation of proflavine completely but denatured DNA and RNA were able to inhibit the photodegradation by around 53% and 60% respectively (Figure 12-B)

Figure 7. Effect of specific hydroxyl radical scavengers on the photogeneration of hydroxyl radical by proflavine.

The reaction mixture contain 10 mM sodium phosphate buffer pH 7.4, 2 mM deoxyribose and 200 μM proflavine and 50 mM of either thiourea or sodium formate or mannitol in a total volume of 0.5 ml. Reaction mixture was incubated for five hours under fluorescent light with proflavine (-◪-) or with proflavine and 100 μM Cu(II) (-⊡-).


100
90
80
70
60
50
40
30
20
10
0
% Inhibition
Control
Thio Urea
Mannitol
Sodium Formate

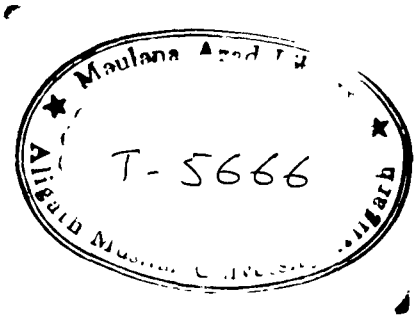


Figure 8. Effect of time on the absorption spectra of proflavine.

Spectra of 20 μM of proflavine in a total volume of 2.0 ml reaction mixture containing 10 mM sodium phosphate buffer, pH 7.4. Spectra were recorded directly after mixing reagents in the spectrophotometer cuvette (Trace 1), and then after incubation in fluorescent light for 30 min (Trace 2), one hour (Trace 3), two hours (Trace 4) and four hours (Trace 5).

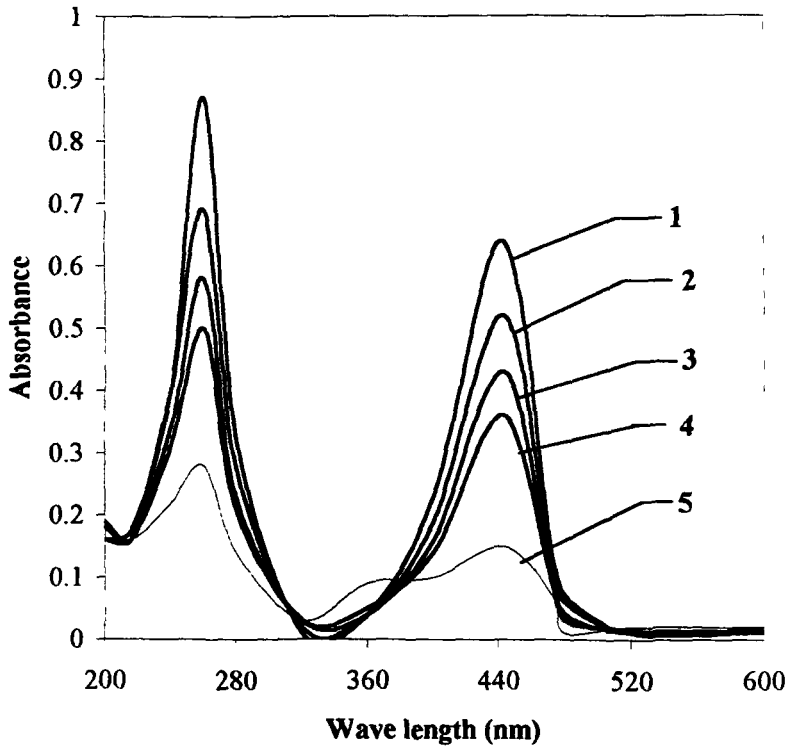

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
200
280
360
440
520
600
Wave length (nm)
1
2
3
4
5

Figure 9. Inhibitions of proflavine degradation by free radical scavengers.

The 2.0 ml reaction mixture contained 10 mM sodium phosphate buffer, pH 7.4, 25 μM proflavine and 50 mM of either sodium azide, potassium iodide, thiourea, mannitol, or 100 μg of superoxide dismutase or catalase. Spectra were recorded after four hours of incubation in fluorescent light.

(-▲-) Proflavine alone at zero time

(-○-) Proflavine + thiourea after four hours.

(-□-) Proflavine + potassium iodide after four hours.

(-X-) Proflavine + sodium azide after four hours.

(-Δ-) Proflavine alone after four hours.

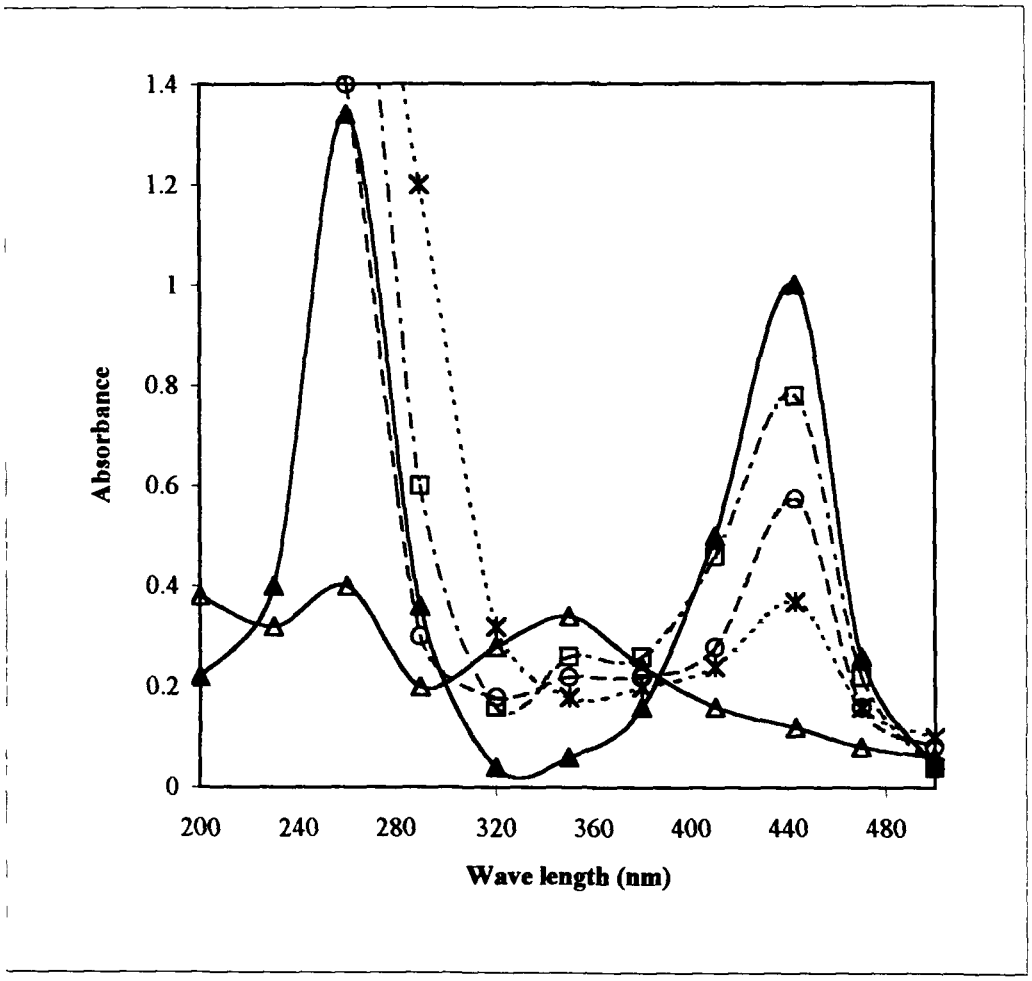

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
200
240
280
320
360
400
440
480
Wave length (nm)

Figure 10. Absorption spectra of proflavine before and after the illumination with fluorescent light in the presence of divalent metal ion Cu(II)

The 2.0 ml reaction mixture contained 10 mM sodium phosphate buffer, pH 7.4, and 200 μM proflavine with or without 100 μM Cu(II).

(-▲-) Proflavine alone at zero time.

(-Δ-) Proflavine + Cu(II) at zero time.

(-●-) Proflavine alone after four hours.

(-○-) Proflavine + Cu(II) after four hours.

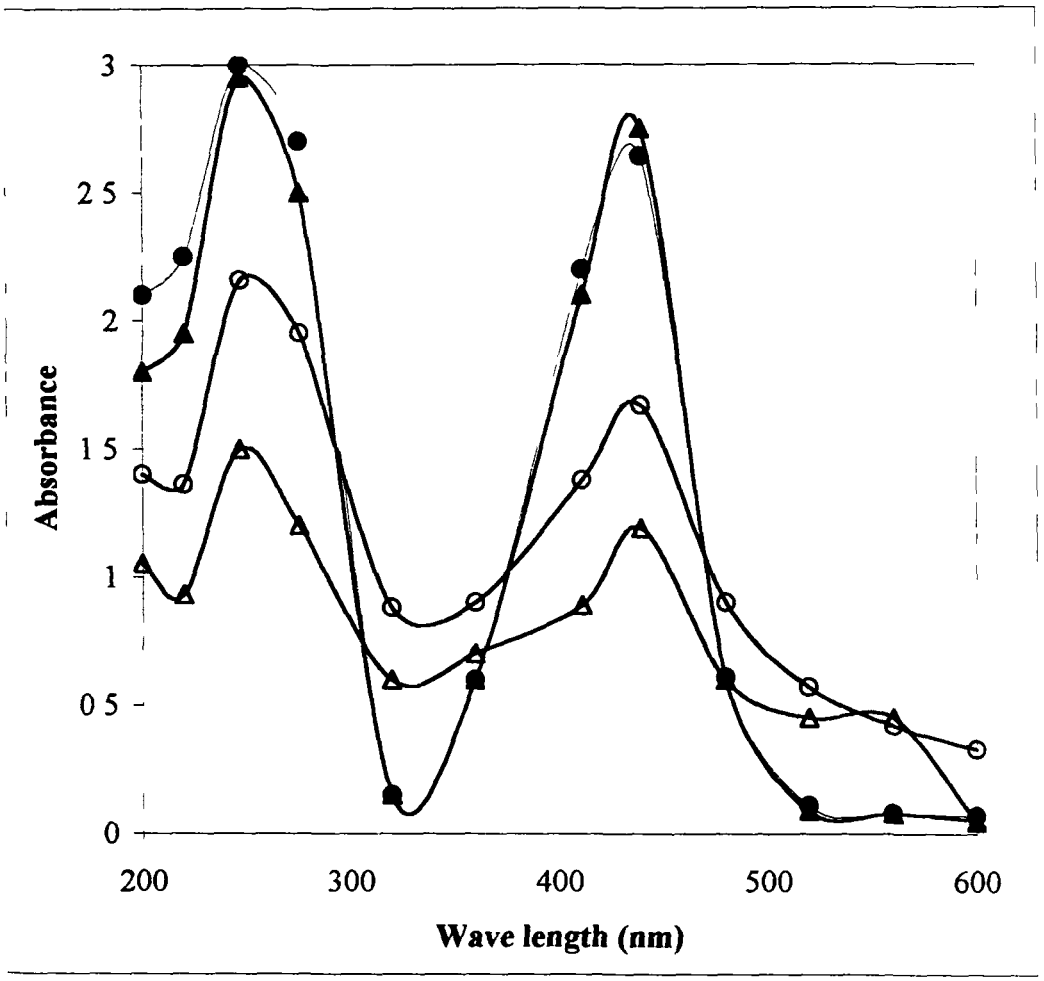

3
2 5
2
1 5
1
0 5
0
Absorbance
200
300
400
500
600
Wave length (nm)

Figure 11. Absorption spectra of proflavine and BSA before (——) and after (.....) illumination with fluorescent light.

The 2.0 ml reaction mixture contained 10 mM sodium phosphate buffer, pH 7.4, 0.1 mg/ml BSA and 50 μM proflavine with or without 25 μM Cu(II).

(-▲-) Proflavine alone at zero time.

(-■-) Proflavine + BSA at zero time.

(-●-) Proflavine + Cu(II) + BSA at zero time.

(-Δ-) Proflavine alone after four hours.

(-❑-) Proflavine +BSA after four hours.

(-○-) Proflavine + Cu(II) + BSA after four hours.

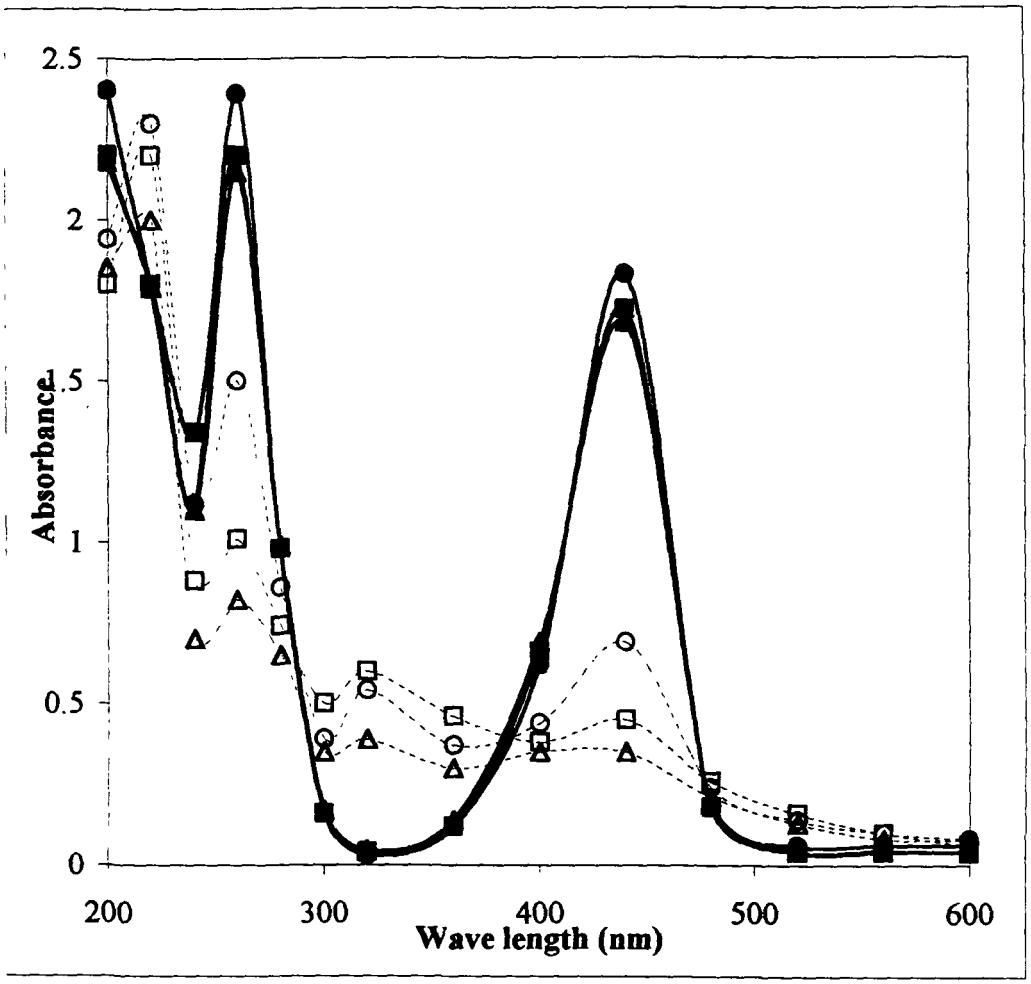

2.5
2
1.5
1
0.5
0
Absorbance
200
300
400
500
600
Wave length (nm)

Figure 12. Absorption spectra of proflavine before and after the illumination with fluorescent light in the presence of DNA (d.s.), DNA (s.s.) and RNA.

The reaction mixture in 2.0 ml contained 10 mM sodium phosphate buffer, pH 7.4, 200 μM proflavine and 200 μg/ml of either DNA (d.s.), DNA (s.s.) or RNA.

(A) Absorption spectra at zero time of photoillumination:

(____) Proflavine alone.

(.....) Proflavine + double stranded DNA.

(-x-x-) Proflavine + single stranded DNA.

(------) Proflavine + RNA

(B) Absorption spectra after four hours of photoillumination:

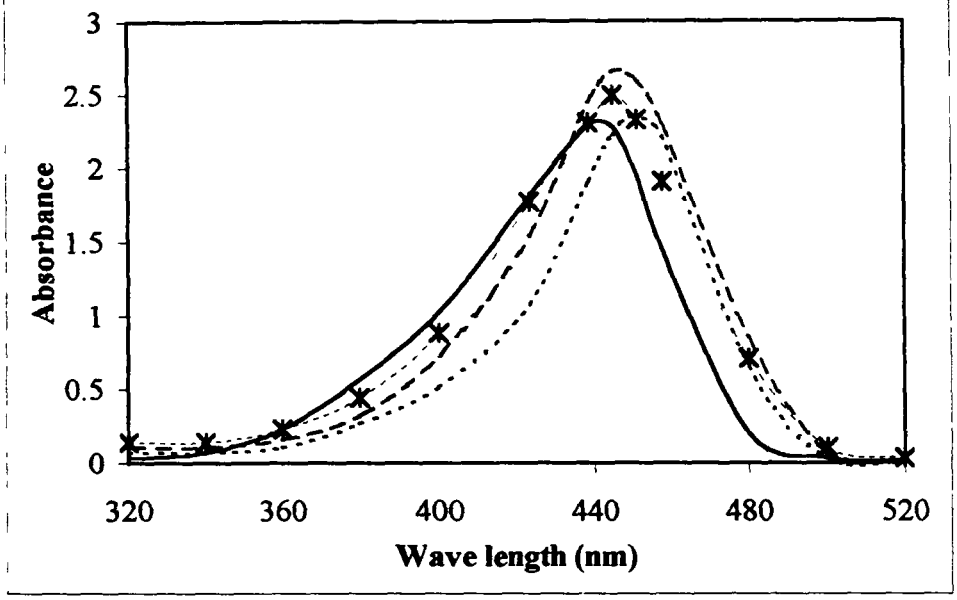
(____) Proflavine alone.

(.....) Proflavine + double stranded DNA.

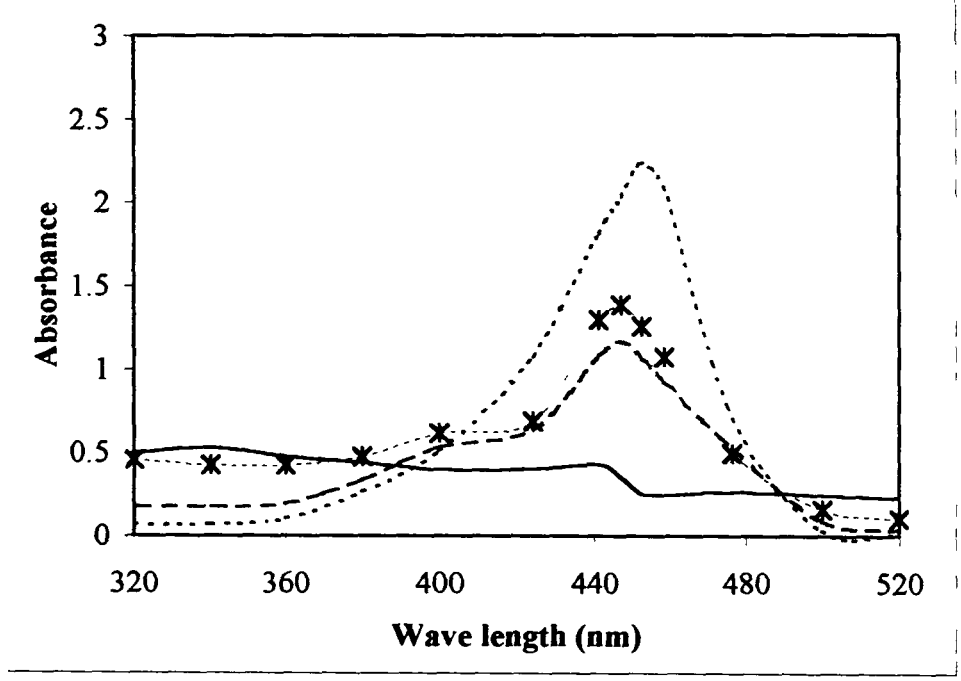
(-x-x-) Proflavine + single stranded DNA.

(------) Proflavine + RNA

A


3
2.5
2
1.5
1
0.5
0
Absorbance
320
360
400
440
480
520
Wave length (nm)

B


3
2.5
2
1.5
1
0.5
0
Absorbance
320
360
400
440
480
520
Wave length (nm)

Effect of various forms of nucleic acid on the production of superoxide anion by photoilluminated proflavine

We further explored whether the extent of $O_2^{\cdot-}$ production by photoilluminated proflavine could be affected by macromolecules. Most of the studies that have been carried out by Piette and co-worker (Peitte *et al.*, 1978; Piette *et al.*, 1979; Piette *et al.*, 1981; Piette *et al.*, 1982) have shown that induction of radical is possible only in the presence of macromolecule such as DNA and this was reported to induce the production of peroxide radical and singlet oxygen. Van de Vorst et al. (1976) however, has suggested the involvement of superoxide and formyl radical in the same photoilluminated reaction mixture. An experiment was carried out using native DNA, denatured DNA and RNA to see their effect on $O_2^{\cdot-}$ production. The production of $O_2^{\cdot-}$ by proflavine was faster in presence of double stranded DNA than in the presence of RNA or single stranded DNA. Both single stranded DNA and RNA were actually inhibitory when compared with the rate in the presence of proflavine alone (Figure 13).

It is now known that intercalation of planar ligands between DNA base pairs involves partial unwinding of the double helix (Waring, 1970). Therefore, interstrand covalent crosslinks are expected to block intercalation. We have used cross-linked dsDNA to study its effect on the proflavine-mediated photogeneration of $O_2^{\cdot-}$. In addition to introducing guanine-guanine and guanine-adenine crosslinks, nitrous acid oxidatively deaminates cytosine, adenine and guanine residues (Singer, 1980). Using such DNA the stimulatory effect on $O_2^{\cdot-}$ formation was not observed (Figure 14). DMS is a monofunctional alkylating agent and weak carcinogen. The major site of alkylation on dsDNA is the N-7 position of guanine (about 75% of total alkylation (Singer, 1983)). Depurinated DNA obtained from DMS alkylated DNA (see Methods) would, therefore, predominantly be depleted in guanine bases. Comparative observation using various modified DNA on the generation of $O_2^{\cdot-}$ by proflavine showed that alkylated DNA give higher stimulatory effect than that of depurinated DNA (Figure 14). This result indicates that proflavine preferentially interacts with guanine bases in dsDNA and hence, enhanced the production of $O_2^{\cdot-}$.

Figure 13. Effect of DNA and RNA on the photogeneration of superoxide anion ($O_2^{\cdot-}$) by proflavine.

Effect of native-, denatured-DNA and RNA on superoxide anion ($O_2^{\cdot-}$) generation by proflavine. Incubation was carried at 25°C for different time intervals on illumination under fluorescent light and with 200 μM proflavine.

(-▲-) Proflavine alone.

(-■-) Proflavine + 200 μg/ml double stranded DNA.

(-◆-) Proflavine + 200 μg/ml single stranded DNA.

(-○-) Proflavine + 200 μg/ml RNA.

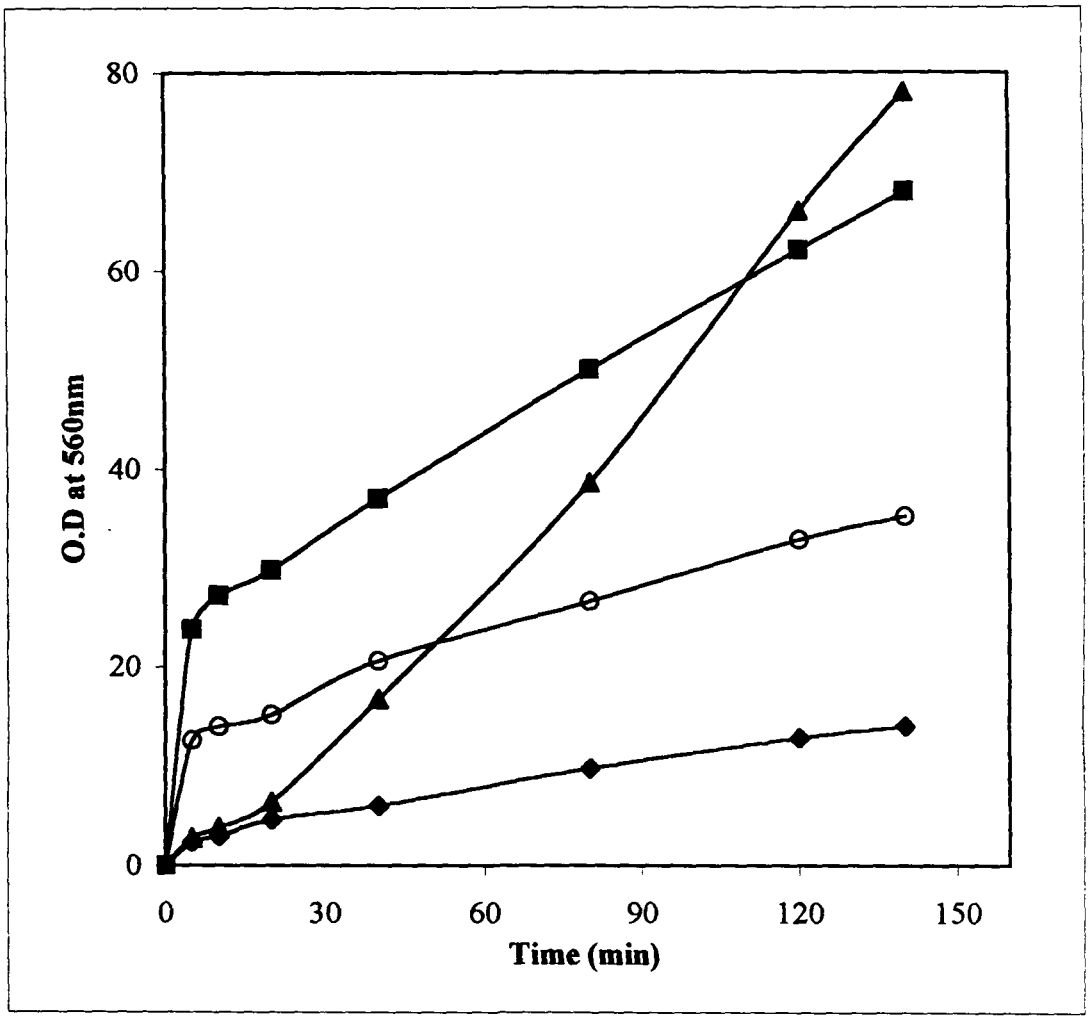

80
60
40
20
0
O.D at 560nm
0
30
60
90
120
150
Time (min)

Figure 14. Effect of modified DNA on the photogeneration of superoxide anion ($O_2^{\cdot-}$) by proflavine.

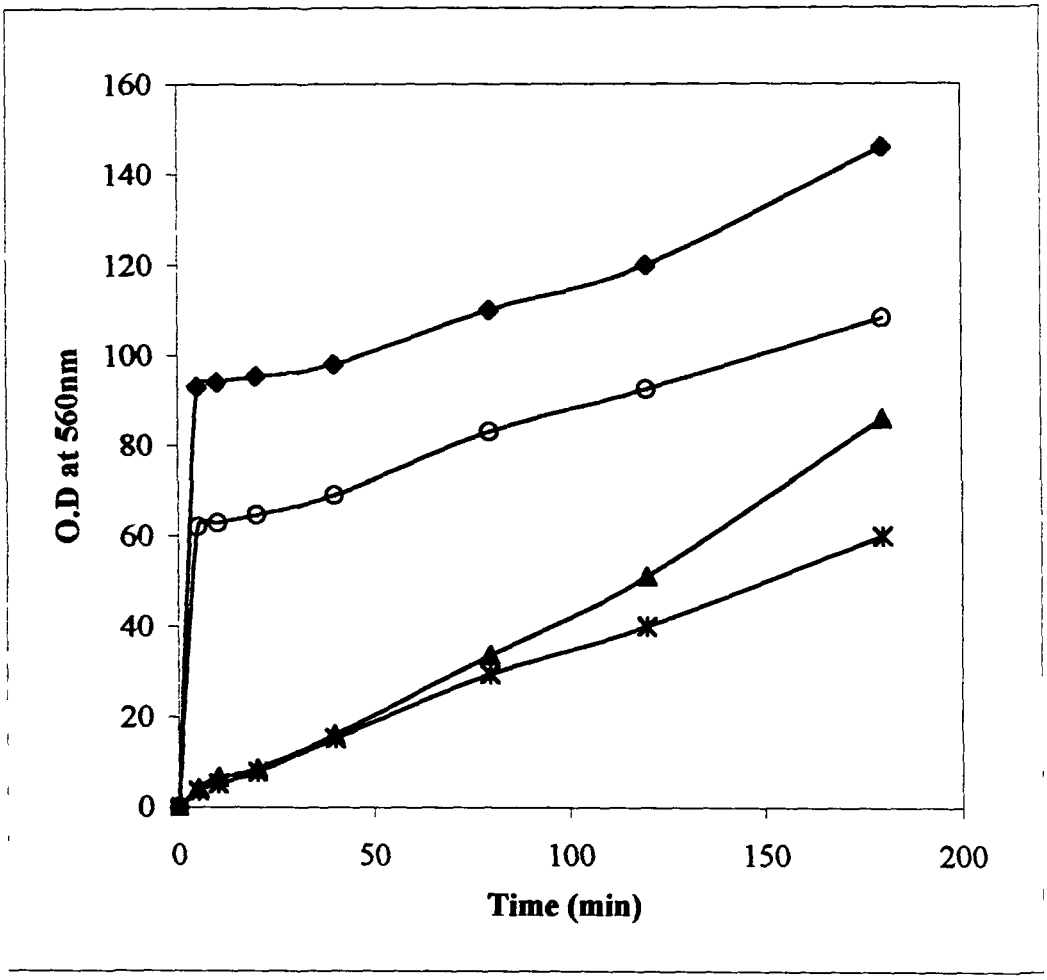
Effect of alkylated, depurinated and cross-linked DNA on $O_2^{\cdot-}$ generation by proflavine. Incubation was carried at 25°C for different time intervals and with 200 μM proflavine after illumination by fluorescent light.

(-▲-) Proflavine alone.

(-■-) Proflavine + 400 μg/ml alkylated DNA.

(-○-) Proflavine + 400 μg/ml depurinated DNA.

(-X-) Proflavine + 400 μg/ml cross-linked DNA.


160
140
120
100
80
60
40
20
0
O.D at 560nm
0
50
100
150
200
Time (min)

Requirement of light for proflavine mediated protein degradation

Wolff and Dean (1986) have reported that radical attack can cause decrease in amino groups and increase in the carbonyl groups, indicating that there is possibility of either increase or decrease in the TCA soluble peptides released after tryptic proteolysis. As photosensitized proflavine is known to bind to DNA and cause strand breaks. It was of interest to examine the proflavine mediated damage to proteins in presence or absence of transition metal ions and visible light. A quantitative analysis with the use of chemical assay for release of acid-soluble peptides was carried out after treatment of the substrate protein with increasing proflavine concentration and light. BSA after irradiation with proflavine or proflavine and Cu(II) in fluorescent light, when treated with trypsin, caused a gradual decrease in the production of acid-soluble material. In the absence of light, however such effect was not seen (Figure 15). Reaction was found to be concentration and time dependent (figure 15 and figure 16). Cu(II) is reported to have specific binding sites on BSA (Neumann and Sass-Kortasak, 1967). Such binding can help in protecting histidyl residues and α–amino group of aspartic acid residues from chemical modifications (Bradshaw *et al.*, 1968), protein fragmentation reaction therefore, was also affected by increasing of Cu(II) (Figure 17). For subsequent experiments, incubation was carried out in 800 lux of fluorescent light.

All the above results point to one of two possibilities, that there are crosslinks in protein after radical attack as proposed by Wolff and Dean (1986), or on the other hand, the sites for trypsin attack, the basic amino acids lysine and arginine, have been modified and hence in both cases the tryptic hydrolysis of the treated protein is decreased. Lysine or arginine are both reported to be lost by modification after radicals attack (Kang *et al.*, 1985; Davies *et al.*, 1987). Furthermore, there are reports that enhanced proteolytic susceptibility requires the modification of at least two histidines per subunit (Rivett and Levine, 1990).

Modification of protein by proflavine with or without Cu(II)

Since proteins are ubiquitous in the cells in high concentrations, modification of proteins by free radicals may lead to disturbance in critical cellular functions. In most cases only qualitative measurements have been made for studying protein modifications

Figure 15. Effect of increasing Proflavine concentration on BSA degradation: Assessment by tryptic proteolysis of BSA.

BSA at 2mg/ml was exposed to increasing concentration of proflavine with or without 100μM Cu(II) in fluorescent light. The TCA soluble product was measured after BSA was treated with 10 μg of trypsin for 30 min.

(-▲-) BSA + Proflavine.

(-●-) BSA + Proflavine + Cu(II).

(-Δ-) BSA + Proflavine in dark.

(-○-) BSA + Proflavine + Cu(II) in dark.

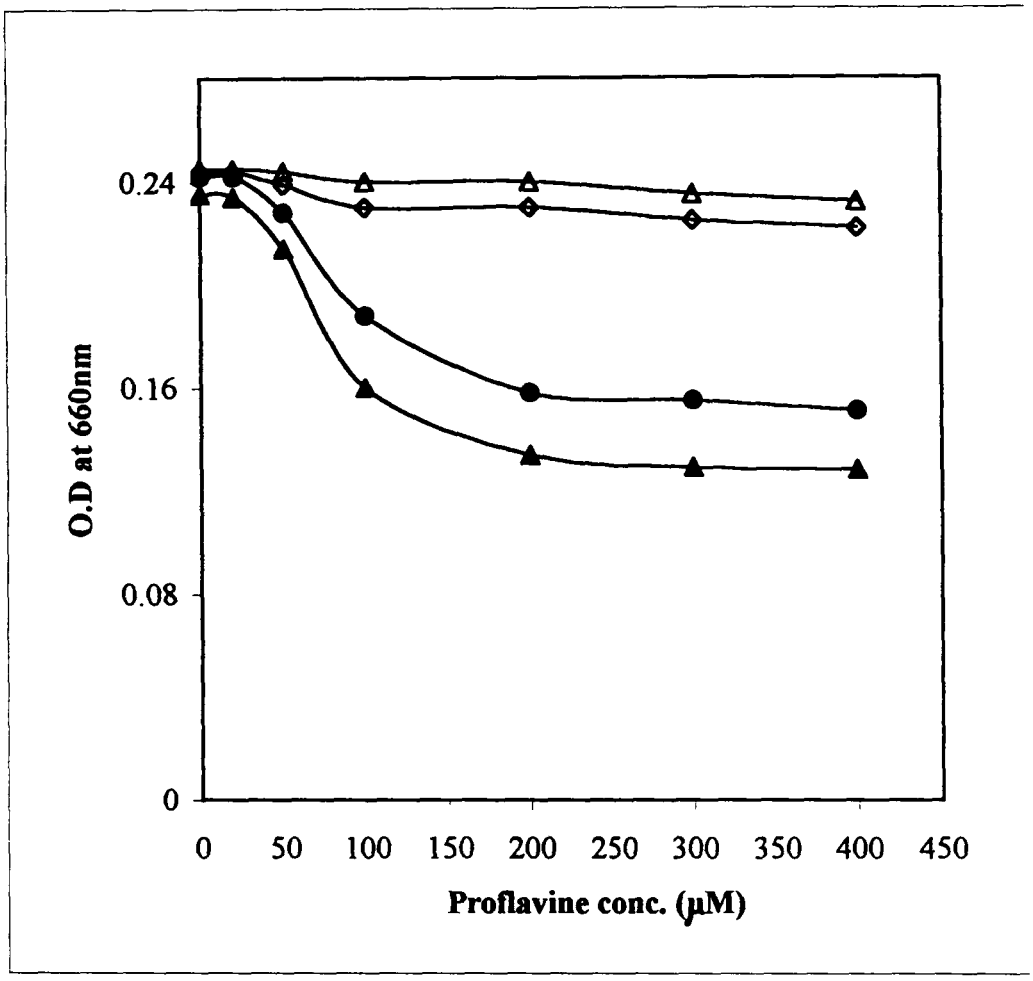

0.24
0.16
0.08
0
O.D at 660nm
0
50
100
150
200
250
300
350
400
450
Proflavine conc. (µM)

Figure 16. Tryptic proteolysis of BSA after incubation with photoilluminated proflavine.

2mg/ml of BSA was exposed to 200 μM of photoexcited proflavine with or without 100 μM Cu(II). After different time intervals, 10 μg of trypsin was added and the reaction mixture incubated at 37°C for 30 min. TCA soluble material was measured as described in the text.

(-▲-) BSA exposed to Proflavine alone.

(-●-) BSA exposed to Proflavine + Cu(II).

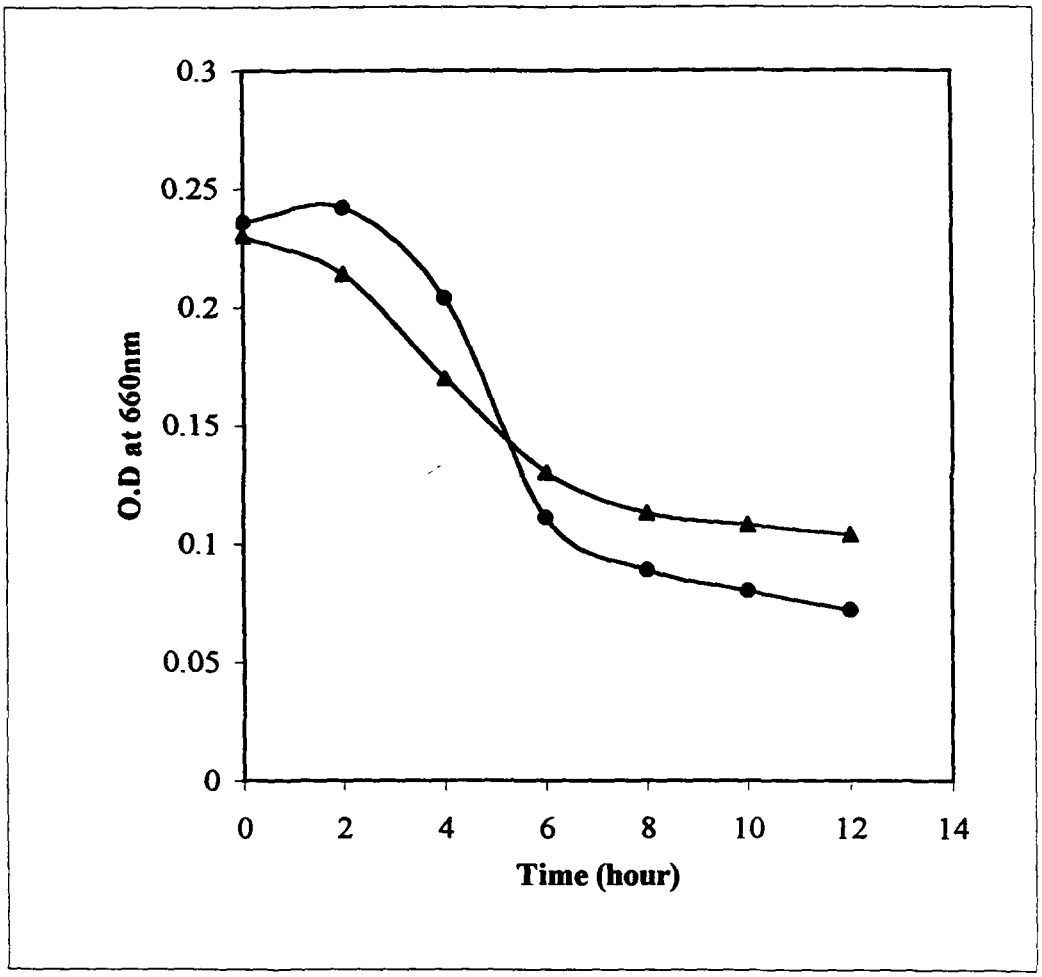
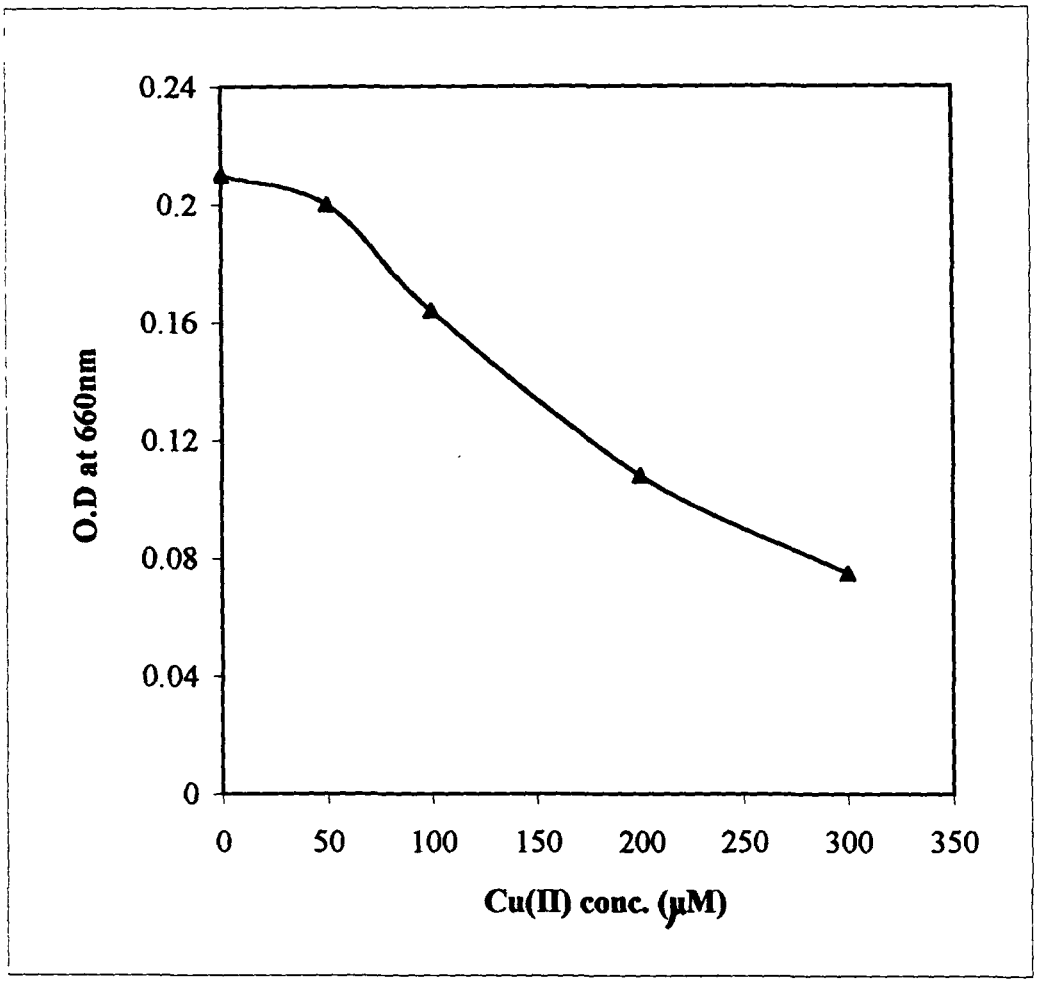

0.3
0.25
0.2
0.15
0.1
0.05
0
O.D at 660nm
0
2
4
6
8
10
12
14
Time (hour)

Figure 17. Effect of increasing Cu(II) concentration on the degradation of BSA by photoilluminated proflavine. Assessment by tryptic proteolysis.

BSA at 2mg/ml was exposed to 200 μM proflavine in the presence of 0, 50, 100, 200 and 300 μM Cu(II) for six hours. The TCA soluble product after tryptic hydrolysis was measured as described in 'Methods'.


0.24
0.2
0.16
0.12
0.08
0.04
0
O.D at 660nm
0
50
100
150
200
250
300
350
Cu(II) conc. (µM)

but quantification by SDS-PAGE under reducing conditions is difficult as the fragments are often small and difficult to retain (during electrophoresis and/or staining). Individual fragments may stain differentially depending on their composition and degree of oxidation (Davies and Delsignore, 1987). To investigate the effect of reactive oxygen species generated by photoilluminated proflavine on proteins, BSA was used as target molecule. The commercial BSA used showed bands of higher molecular weight, in addition to the BSA monomer of 67 kDa. These bands may represent aggregates of BSA and have been previously reported by other workers (Hunt *et al.*, 1988). BSA was incubated with increasing concentrations of proflavine alone (Figure 18-A) or in presence of Cu(II) (Figure 18-B). The SDS-PAGE profile of BSA after exposure to proflavine and light showed significant degradation. The cleavage of the large aggregates present in the commercial sample, that migrate near the top of the gels was decreased followed by the broadening of the band at monomer position after five hours of irradiation in fluorescent light. This is partially due to the overlapping, the staining system and monomer fragmentation to slightly smaller peptides. Presence of Cu(II) resulted in enhanced protein degradation to smaller fragments (Figure 18-B). It is well reported that with metal-ion-catalysed oxidations the location of the active transition-metal ion become important, and residues such as histidine, cystine and methionine, which bind metal ions, may localize reactions to their vicinity (Bachur *et al.*, 1979; Levine *et al.*, 1981). The differential capacities of proteins to bind metal ions and render them either redox-inactive or active also influence the distribution of damage among protein populations. Low concentration of Cu(II) (50 μM), did not result in very pronounced degradation (Figure 19 lane c). Increase in the concentration of Cu(II) however, enhanced the degradation of protein significantly (Figure 19 lane d, e, f). We also confirmed that the release of such peptides from BSA following photoillumination with proflavine was time dependent (Figure 20 A and B). Of the various other metal ions tested only Fe^{3-} induced slight degradation. No degradation of BSA by either proflavine in absence of light or by Cu(II) alone was observed (data not shown). All our experiments were conducted in phosphate buffer since other buffers such as Tris, HEPES and carbonate were reported to be effective inhibitors of BSA modification by free radicals (Davies and Delsignore, 1987) or it can give unwanted radicals (Halliwell and Gutteridge, 1985).

Figure 18. SDS-polyacrylamide gel electrophoresis of BSA after incubation with proflavine and Cu(II).

Electrophoresis was performed on 10% SDS-polyacrylamide gel and 10 μg of protein was applied in each lane after the following treatment was given to the protein. Gels were later silver stained as described in 'Methods'.

(A) Increasing proflavine concentration.

Lane a - Control (BSA incubated alone).

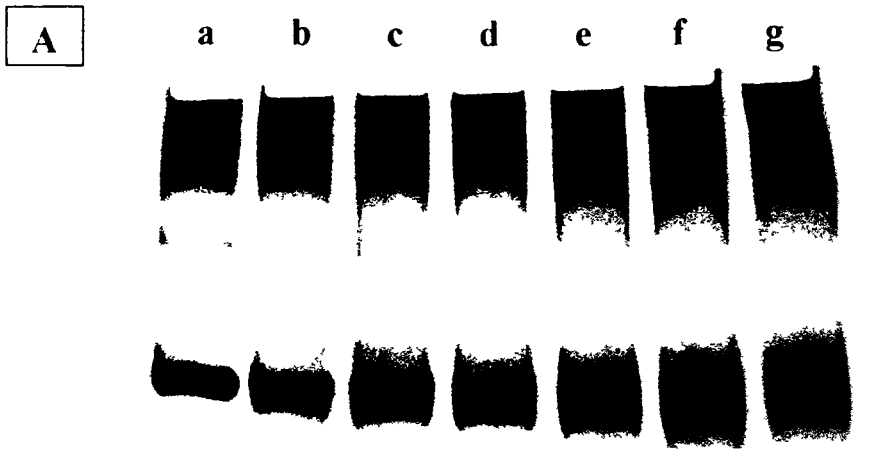
Lane b-g - BSA incubated with 10, 20, 50, 100, 200 and 300 μM proflavine.

(B) Increasing proflavine concentration in the presence of 200 μM Cu(II).

Lane a - Control (BSA incubated alone).

Lane b-g - BSA incubated with 10, 20, 50, 100, 200 and 300 μM proflavine in presence of 200 μM Cu(II).

The reaction mixtures were incubated for six hours in fluorescent light at room temperature before electrophoresis.


A
a
b
c
d
e
f
g

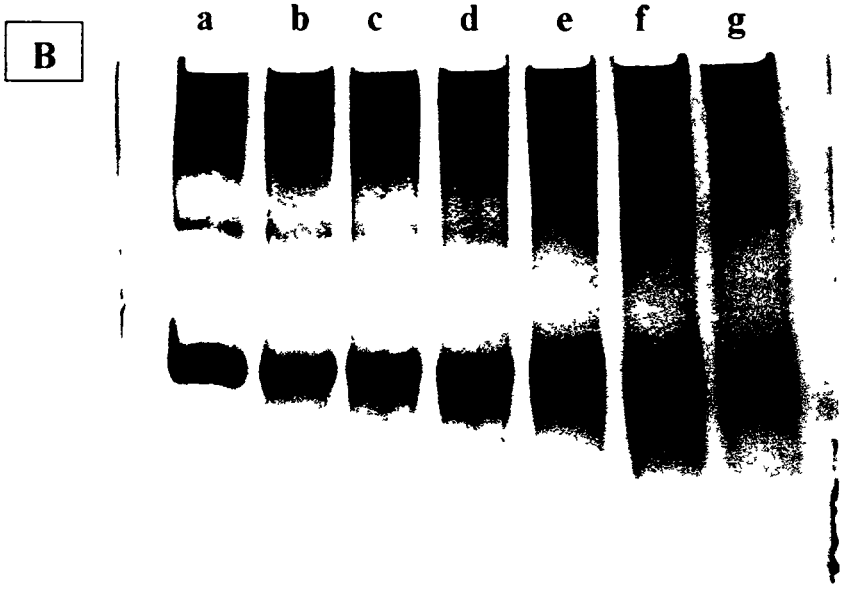

B
a
b
c
d
e
f
g

Figure 19. SDS-polyacrylamide gel electrophoresis of silver stained BSA incubated with increasing concentration of Cu(II) in presence of proflavine.

Electrophoresis was performed on 10% SDS-polyacrylamide gel as described in 'Methods'. 10 μg of protein was applied in each lane after reactions were incubated for six hours in fluorescent light at room temperature. Gels were silver stained after electrophoresis.

Lane a - Control (BSA incubated alone).

Lane b-f - BSA incubated with 200 μM proflavine in presence of 0, 50, 100, 150 and 200 μM Cu(II).

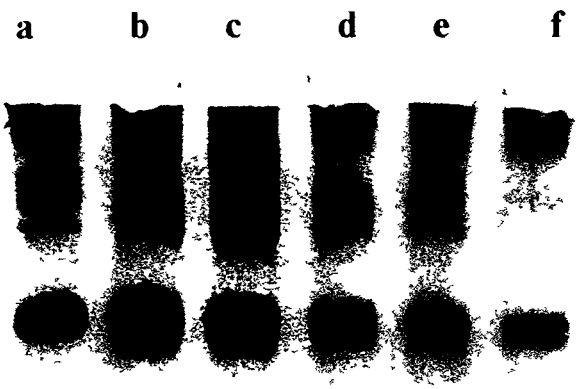

a b c d e f

Figure 20. SDS-polyacrylamide gel electrophoresis of silver stained BSA incubated with proflavine and Cu(II).

Electrophoresis was performed on 10% SDS-polyacrylamide gel and 10 μg of protein was applied in each lane after the following treatment given to protein. Gels were later silver stained as described in 'Methods'.

(A) Effect of increasing time of incubation with proflavine alone.

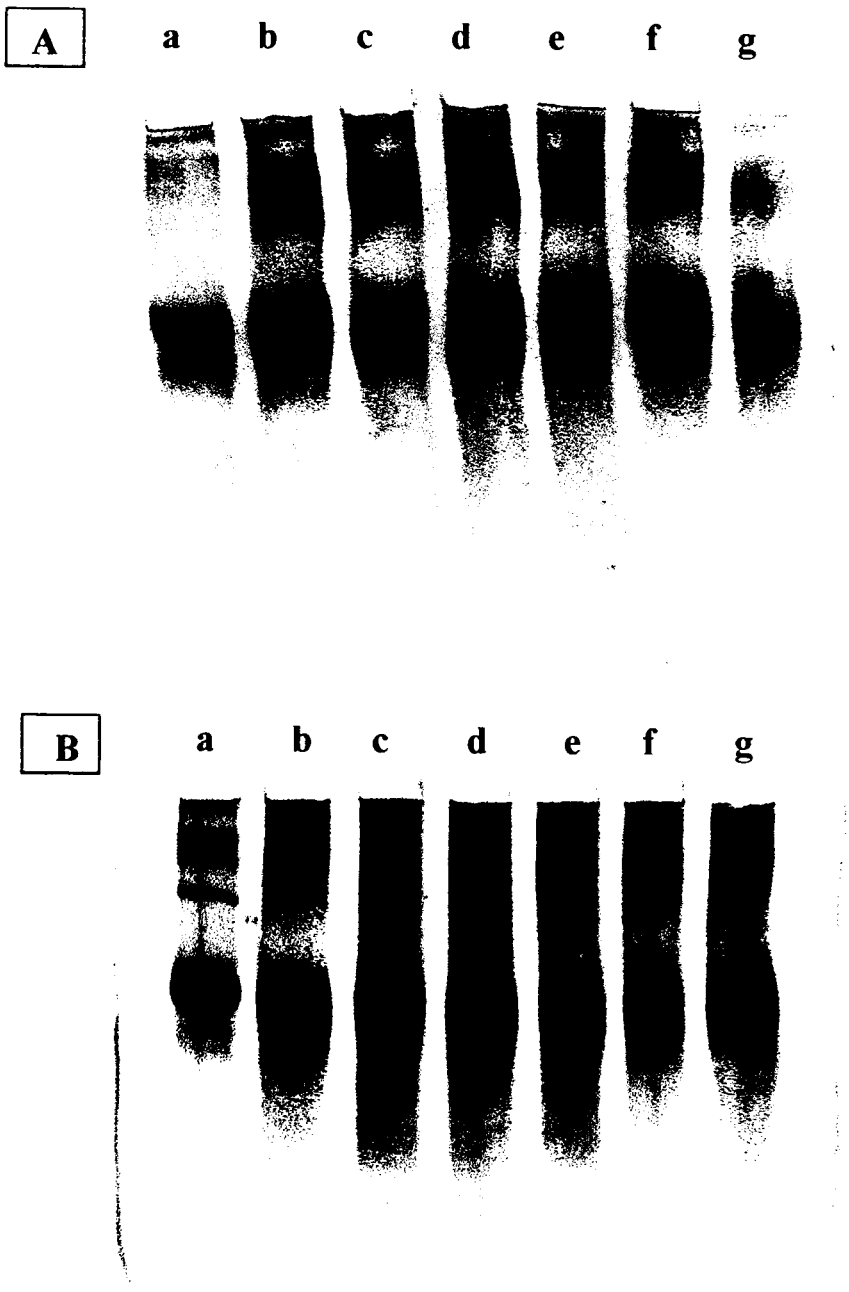
Lane a - Control (BSA incubated alone).

Lane b-g - BSA incubated for 2, 4, 6, 8, 10 and 12 hours with 200 μM proflavine.

(B) Effect of increasing time of incubation with proflavine in the presence of Cu(II).

Lane a - Control (BSA incubated alone).

Lane b-g - BSA incubated for 2, 4, 6, 8, 10 and 12 hours with 200 μM proflavine in presence of 200 μM Cu(II).


A
a b c d e f g
B
a b c d e f g

Involvement of oxygen free radicals and Cu(I) on protein damage mediated by proflavine and proflavine-Cu(II) when illuminated with fluorescent light

Previous studies by Piette and co-worker workers (Peitte *et al.*, 1978; Piette *et al.*, 1979; Piette *et al.*, 1981; Piette *et al.*, 1982) have demonstrated that proflavine upon illumination with visible light gets activated and generate various free radicals only in presence of macromolecules like DNA. The target molecule in our study is BSA. BSA was reported to yield fragments of different sizes after exposing it to $^{\cdot}OH$ in the presence of O_2. In absence of O_2 however, $^{\cdot}OH$ caused substantial crosslinking (Wolff and Dean, 1986; Davies, 1987). Furthermore, the work done by Davies (1987) have shown that exposure to $O_2^{\cdot-}$ caused no change in molecular weight of any protein, and exposure to $^{\cdot}OH$ alone can induce a generalized aggregation of proteins to higher molecular weight forms. He have also reported that exposure to $^{\cdot}OH$ plus $O_2^{\cdot-}$, generally produced low molecular weight protein fragments (Davies, 1987).

The effect of various free radical scavengers on protein degradation by proflavine such as sodium azide, potassium iodide, thiourea were studied in presence of O_2 (Figure 21). Potassium iodide showed a significant inhibitory effect on BSA degradation. Sodium azide and thiourea were also effective in inhibiting the protein degradation (Figure 21-A). A similar pattern of inhibition by scavengers was observed when Cu(II) was also present in the reaction (Figure 21-B). All these results point to a mechanism involving singlet, triplet oxygen and hydroxyl radical.

In order to investigate whether the sequestration of Cu(I) results in abolition of BSA fragmentation. Bathocuproine, a specific Cu(I) sequestering agent was also used. When included in the reaction containing Cu(II), bathocuproine at a concentration of 200 μM significantly inhibited the protein degradation (Figure 21-B lane g), confirming the involvement of Cu(I) in the reaction, showing the stoichiometry of bathocuproine-Cu(I) complex as 2:1.

Enzyme inactivation by photoilluminated proflavine

Previous studies of Gantchev and Van Lier (1995) have shown that exposure of catalase (from bovine liver) to the photosensitizer like tetrasulfonated metasllophthalocyanines caused conformational changes in the enzyme. The activity of

Figure 21. Inhibition by various radical scavengers in BSA degradation induced by proflavine.

Electrophoresis was performed on 10% SDS-polyacrylamide gel as described under 'Methods'. 10 μg of protein was applied in each lane, after the following treatment was given to the protein. Gels were later silver stained after completion of electrophoresis.

(A) BSA incubated with 200 μM proflavine:

(lane a) BSA alone.

(lane b) BSA incubated with proflavine.

(lanes c, d, e) BSA, proflavine and 50 mM of sodium azide, potassium iodide or thiourea respectively.

(B) BSA incubated with 200 μM proflavine and 200 μM Cu(II):

(lane a) BSA alone.

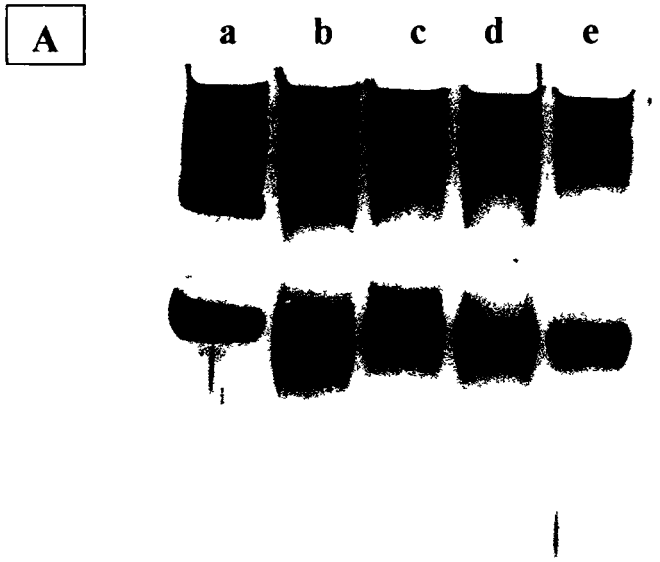
(lane b) BSA with proflavine.

(lane c) BSA with proflavine and Cu(II).

(lanes d, e, f) BSA, proflavine and 50 mM of sodium azide, potassium iodide or thiourea respectively.

(lane g) represent bathocuproine with a molar ratio to Cu(II) of 2:1.

In both **(A)** and **(B)** the reactions were incubated for six hours in fluorescent light at room temperature.


A
a
b
c
d
e


B
a
b
c
d
e
f
g

catalase decreased when exposed to various photosensitizers. Various reactive species are known to participate in photosensitizer induced inactivation of enzymes as reported on purified catalase, or catalase within cells (Gantchev and Lier, 1995). We tested above finding using our system of the photoilluminated proflavine with or without Cu(II) on two enzymes with different physiological and structural properties namely invertase, a heavily glycosylated protein, and trypsin a well known proteolytic enzyme. Glycoproteins are widely distributed and varied products of post-transnational modification, most of them are either secretary proteins or are important components of membranes. In a heavily glycosylated protein like invertase the peptides are likely to be buried inside and hence, expected to show resistance to damage cause by various reactive oxygen species. As reactive oxygen species are known to attack amino acids with preference to lysine, arginine, and proline residues (Stadtman, 1991). Invertase was used as target to test the shielded amino acids. Cross et al. (1984) have shown that glycopolypeptides are effective scavengers of ·OH when they are generated in the system due to the presence of attached carbohydrate.

Trypsin is extensively studied protease of serine class. It is widely used in biochemical studies because of its broad substrate specifications. Since proflavine is known for its affinity for serine proteases (Brantner *et al.*, 1976) trypsin was used in the present study.

(A) Invertase study

Catalytic analysis

Yeast invertase, β–D-Fructofuranosidase [EC 3.2.1.26], exists in two distinct forms, the non glycosylated internal and the heavily glycosylated external enzyme (Gascon et al., 1968). The later was our target of study. Photodynamic inactivation of invertase by proflavine with or without Cu(II) was performed (Figure 22). The decrease in the intensity of color at 540 nm as a result of decrease in the liberated glucose from the hydrolysis of sucrose indicated the enhance rate of inactivation of the invertase (Figure 22). After four hours of incubation of enzyme with proflavine and light, the activity was about 43% of the control. However, in presence of Cu(II) the inactivation was faster as before completion of an hour in fluorescent light, the activity of invertase decreased to 20% of the control (Figure 22). The reaction with proflavine alone as well as with Cu(II) was time and concentration dependent (figure 22 and figure 23).

Figure 22. Invertase inactivation by proflavine and Cu(II).

Reaction mixture in a total volume of 0.3 ml contained 100 μg of invertase, 200 μM of proflavine with or without 100 μM Cu(II). Reaction mixtures were incubated at 25°C for different time intervals in fluorescent light. Enzyme activity was assayed as described in 'Methods'.

(-◆-) Invertase alone.

(-X-) Invertase + Cu(II)

(-▲-) Invertase + proflavin.

(-■-) Invertase + proflavine + Cu(II)

(-❑-) Invertase + proflavine + Cu(II) + 110 μM EDTA.

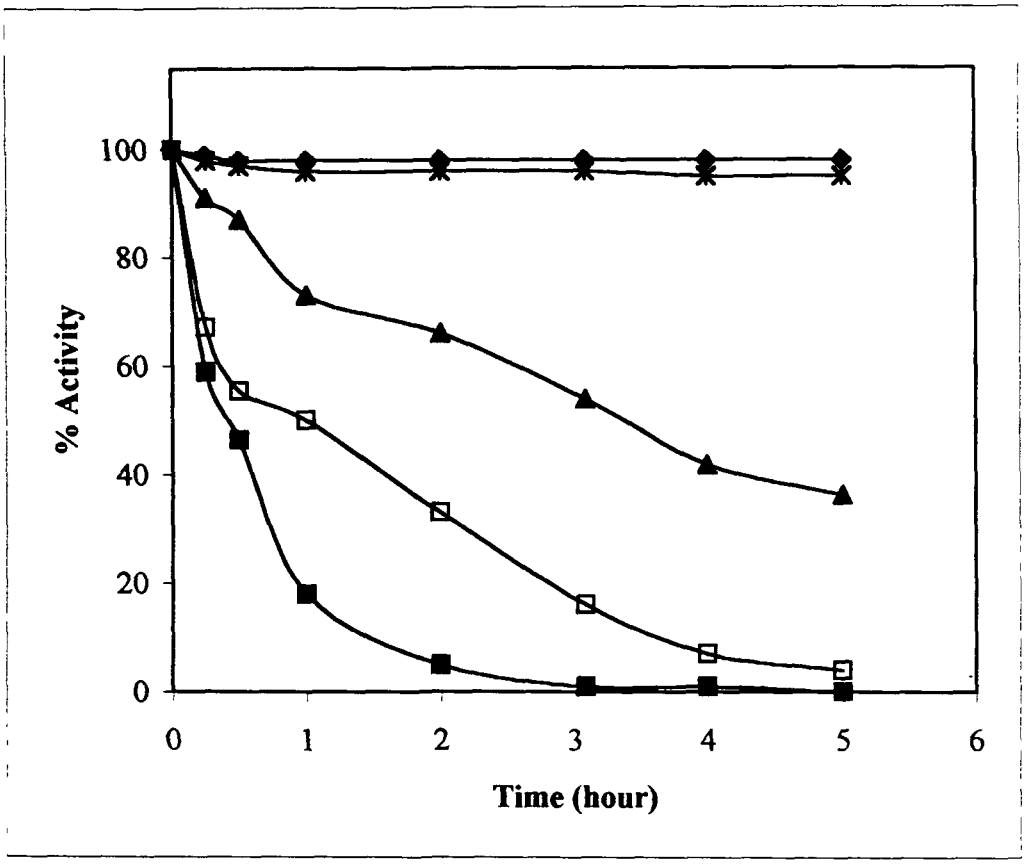

100
80
60
40
20
0
% Activity
0
1
2
3
4
5
6
Time (hour)

Figure 23. Invertase inactivation as a function of increasing proflavine concentration.

Reaction mixture in a total volume of 0.3 ml containing 100 μg of invertase, 10 mM sodium phosphate buffer (pH 7.4) and increasing concentration of proflavine with or without 100 μM Cu(II). Reactions were incubated at 25°C for three hours under fluorescent light. Enzyme activity was assayed as described in 'Methods'.

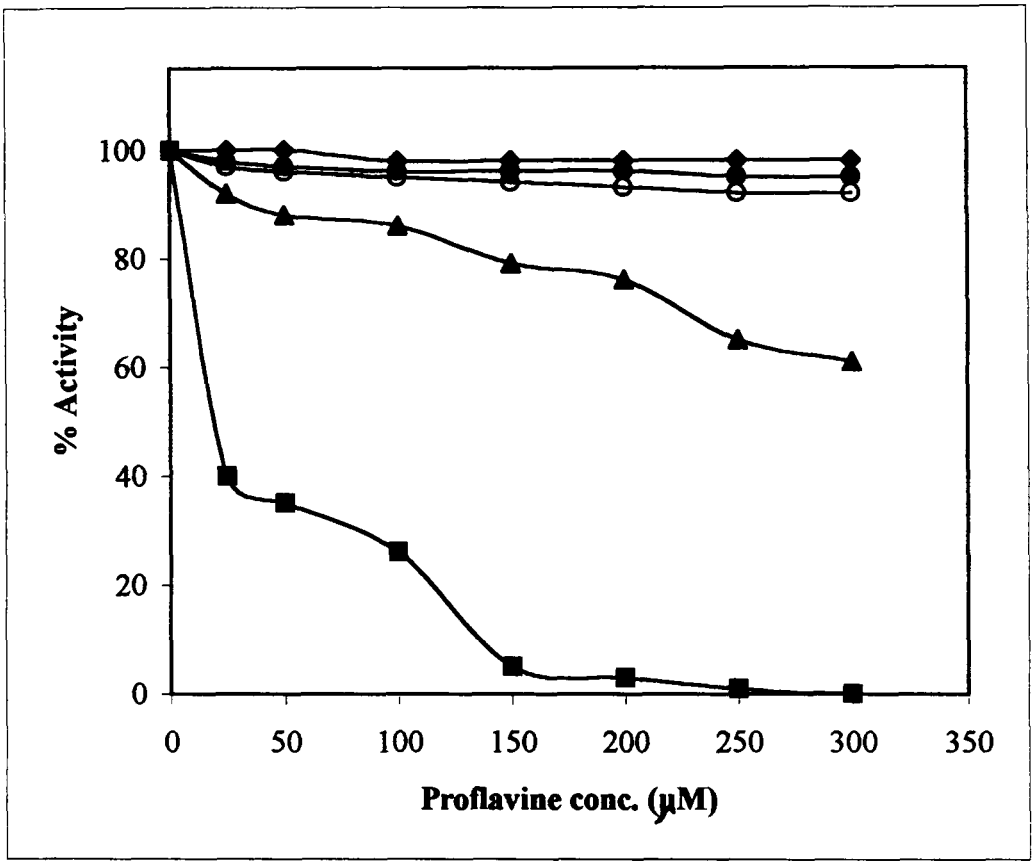
(-◆-) Invertase alone.

(-▲-) Invertase + proflavine.

(-■-) Invertase + proflavine + Cu(II)

(-●-) Invertase + proflavine in dark.

(-○-) Invertase + proflavine + Cu(II) in dark.


100
80
60
40
20
0
% Activity
0
50
100
150
200
250
300
350
Proflavine conc. (μM)

Metal-catalysed oxidation systems are reported to inactivate a wide range of enzymes (Bauskin et al., 1991; Stadtman, 1991). Metal such as Cu(II) is known to inactivate the invertase. The effect of metal ion can be blocked by the addition of metal binding agents such as EDTA (Bernfeld, 1955). EDTA when included in the reaction did not prevent the participation of metal ion in inactivation of enzyme caused by proflavine (Figure 22). Addition of Cu(II) to the photoilluminated reaction caused a rapid decrease in the activity of invertase as the molar ratio of Cu(II) to proflavine increased within four hours (Figure 24). In order to investigate whether the sequestration of Cu(I) results in the recovery of invertase activity, bathocuproine, a specific Cu(I) sequestering agent was included in the reaction. Bathocuproine when included in the reaction containing Cu(II), significantly inhibited the enzyme inactivation (Figure 25) confirming the involvement of Cu(I) in the inactivation reaction.

Electrophoretic Analysis

The above results were further confirmed by gradient polyacrylamide gel electrophoresis. The photosensitized proflavine has induced fragmentation of enzyme in a time dependent manner (Figure 26). Fragmentation was found to be greater when proflavine and Cu(II) were used, as the band started to disappear due to protein degradation (Figure 26 lane e, f, g). Wolff and co-workers (Wolff and Dean, 1987; Wolff et al., 1989; Hunt et al., 1988) have proposed that the hydroxyl radical and Cu(II) catalyzed auto-oxidation of the sugars which will then participate in the modification of amino acids in a protein. Involvement of free radical when proflavine or proflavine and Cu(II) were used with enzyme is demonstrated on gradient PAGE. Scavengers sodium azide, potassium iodide and thiourea gave effective inhibition (Figure 27 A and B).

(B) Trypsin Study

Catalytic analysis

Trypsin [EC 3.4.21.4], is a proteolytic enzyme with a molecular weight lower than invertase. It is a non glycosylated protein with single polypeptide as compared to invertase which has four subunits. The enzyme inactivation is monitored as decreased in release of acid soluble peptides from the substrate. Trypsin was irradiated with fluorescent light and proflavine prior to its use on BSA (as a substrate) (Figure 28). The effect could be seen in a very short time of incubation, that is in 35 min the activity of

Figure 24. Invertase inactivation by proflavine as a function of increasing Cu(II) concentration.

Reaction mixture in a total volume of 0.3 ml contained 100 μg of invertase, 10 mM sodium phosphate buffer (pH 7.4), 200 μM proflavine and increasing concentration of Cu(II). Reactions were incubated at 25°C for three hours under fluorescent light. Enzyme activity was assayed as described in 'Methods'.

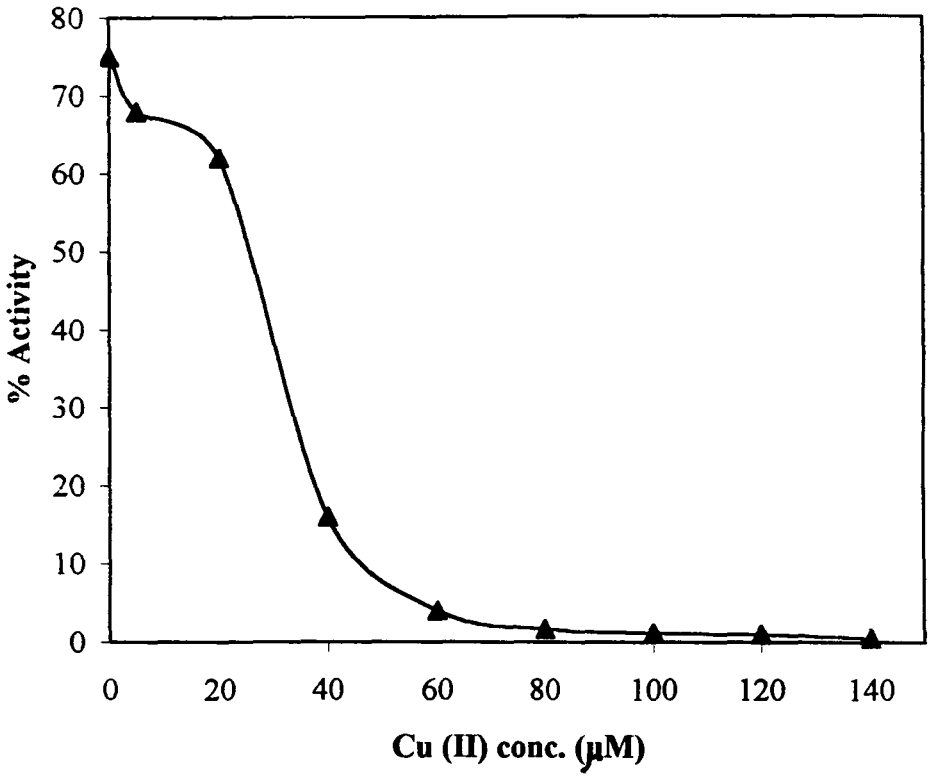

80
70
60
50
40
30
20
10
0
% Activity
0
20
40
60
80
100
120
140
Cu (II) conc. (µM)

Figure 25. Inhibition to proflaivne-Cu(II) induced inactivation of Invertase by bathocuproine.

Reaction mixture in a total volume of 0.3 ml contained 100 μg of invertase, 10 mM sodium phosphate buffer (pH 7.4), 200 μM proflavine, 100 μM Cu(II) and increasing concentration of bathocuproine.

Reactions were incubated at 25°C for three hours under fluorescent light. Enzyme activity was assayed as described in 'Methods'.

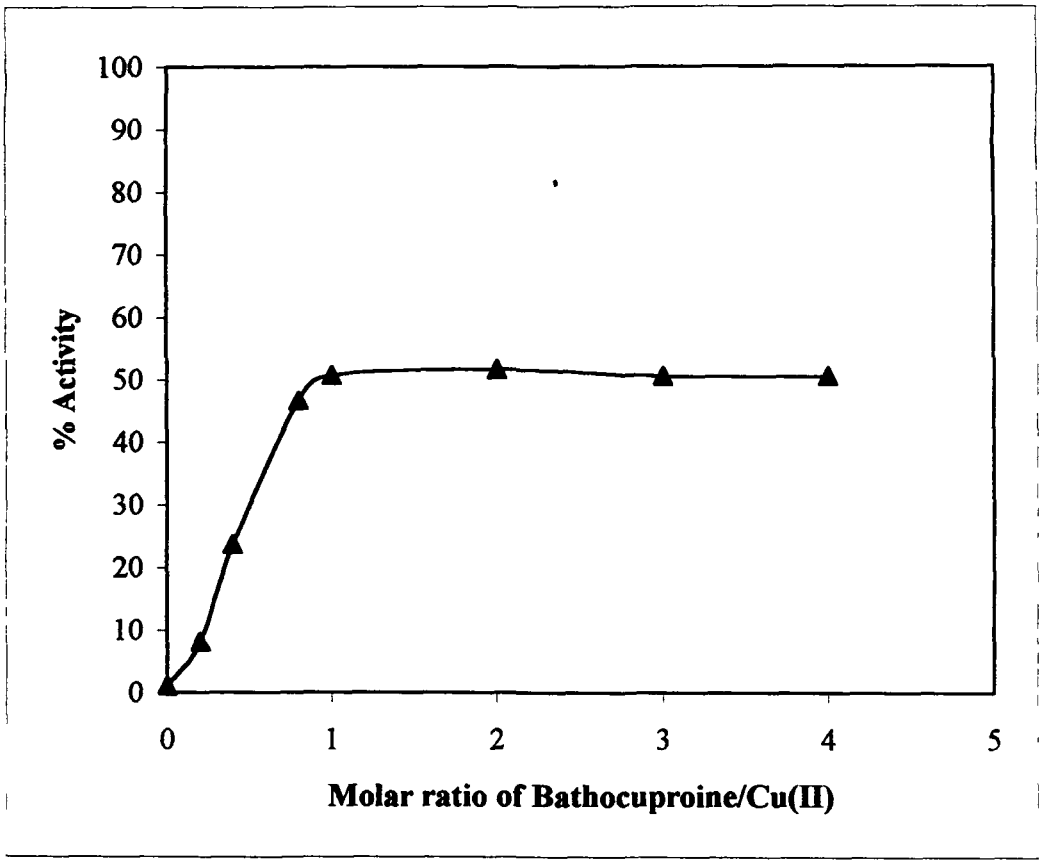

100
90
80
70
60
50
40
30
20
10
0
% Activity
0
1
2
3
4
5
Molar ratio of Bathocuproine/Cu(II)

Figure 26. Gradient polyacrylamide gel electrophoresis of silver stained Invertase incubated with proflavine and Cu(II).

Electrophoresis was performed on 5-12.5% gradient polyacrylamide gel as described in 'Methods'. 80 μg of protein was applied in each lane after the following treatments.

Lane a - Control (Invertase incubated alone).

Lane b-d - Invertase incubated for 2, 4 and 8 hours with 200 μM proflavine.

Lane e-g - Invertase incubated for 2, 4 and 8 hours with 200 μM proflavine and 100μM Cu(II).

The reaction mixture (1 ml) contained 2 mg/ml Invertase, 10 mM sodium phosphate buffer (pH 7.4) and indicated concentrations of proflavine and Cu(II). Incubation was carried out at 25°C for the indicated time intervals. Gels were silver stained after electrophoresis.

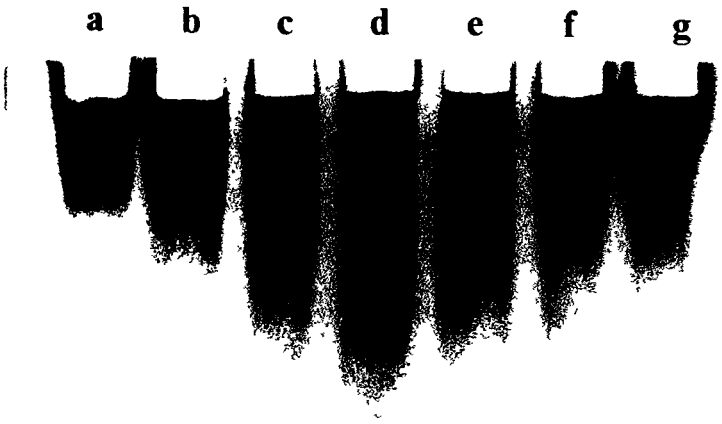

a
b
c
d
e
f
g

Figure 27. Inhibition by radical scavengers of proflavine induced degradation of Invertase.

Electrophoresis was performed on 5-12.5% gradient polyacrylamide gel as described under 'Methods' and 80 μg of protein was applied in each lane.

(A) Invertase incubated with 200 μM proflavine:

(lane a) Invertase alone.

(lane b) Invertase incubated with proflavine.

(lanes c, d, e) Invertase, proflavine and 50 mM of sodium azide or potassium iodide or thiourea respectively.

(B) Invertase incubated with 200 μM proflavine and 200 μM Cu(II):

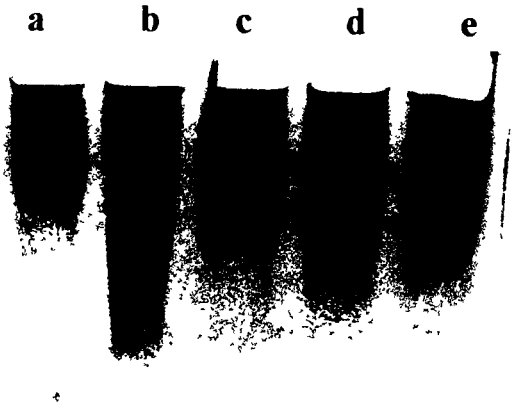
(lane a) Invertase alone.

(lane b) Invertase with proflavine.

(lane c) Invertase with proflavine and Cu(II).

(lanes d, e, f) 50 mM of sodium azide or potassium iodide or thiourea respectively with Invertase, proflavine and Cu(II) system.

In both **(A)** and **(B)** the reactions were incubated for six hours in fluorescent light at room temperature. Gels were silver stained after electrophoresis.


a
b
c
d
e

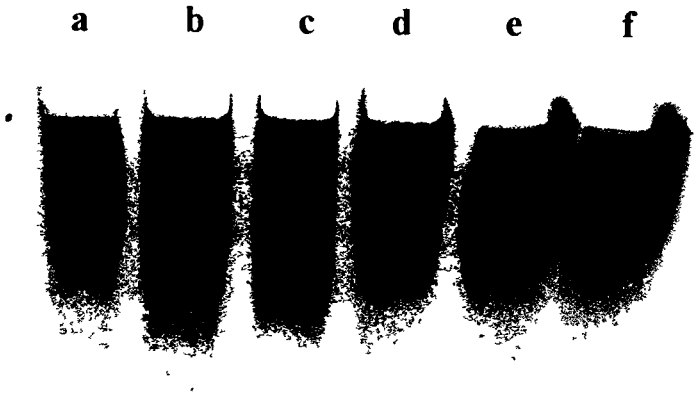

a
b
c
d
e
f

Figure 28. Trypsin inactivation by proflavine and Cu(II)

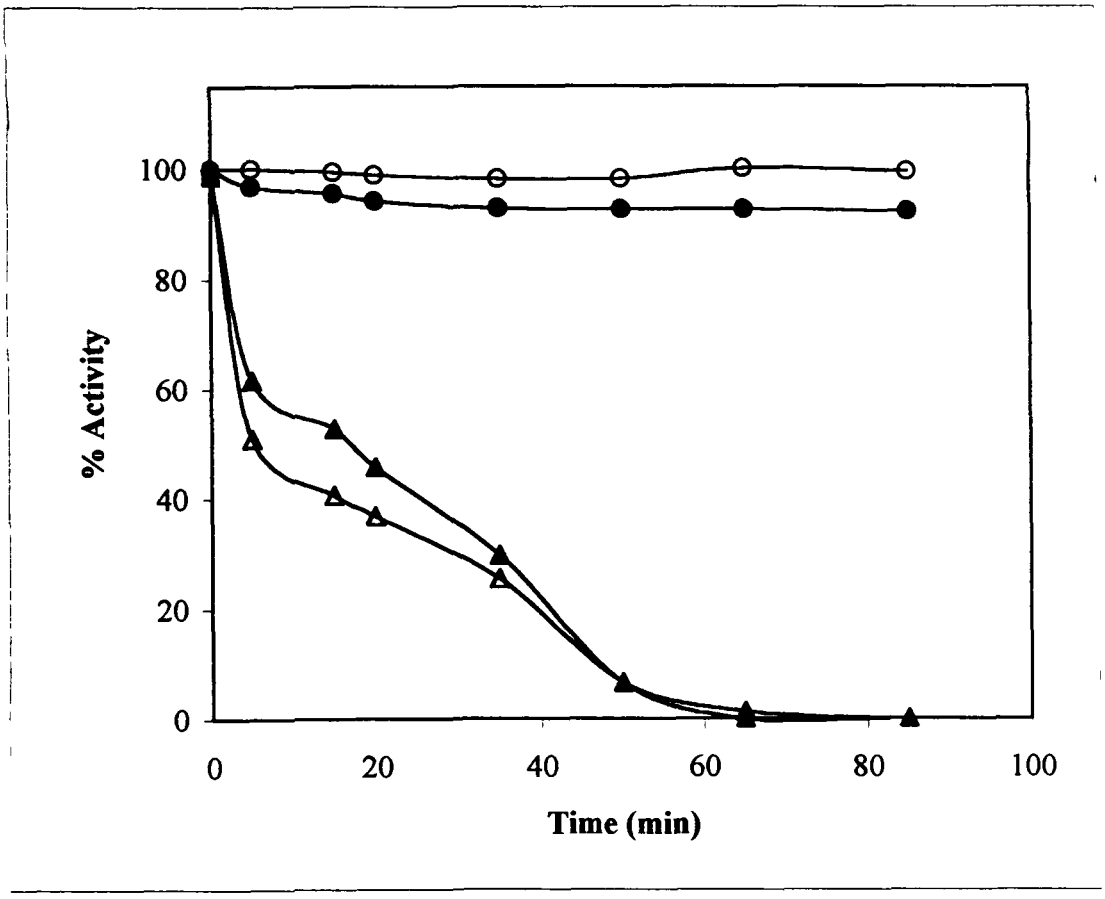
Reaction mixture in a total volume of 2 ml contained 15 μg of trypsin, 10 mM sodium phosphate buffer pH 7.4 and 200 μM of proflavine with or without 100 μM Cu(II). Reactions were incubated at 25°C for different time intervals in fluorescent light. Enzyme activity was assayed as described in 'Methods'.

(-○-) Trypsin alone.

(-●-) Trypsin + Cu(II)

(-▲-) Trypsin + proflavine.

(-Δ-) Trypsin + proflavine + Cu(II)


100
80
60
40
20
0
% Activity
0
20
40
60
80
100
Time (min)

the enzyme was less than 40%. The effect was concentration dependent (Figure 29). Addition of Cu(II) to the photoilluminated reaction caused a rapid decrease as the molar ratio of Cu(II) to proflavine increased within a period of 35 min (Figure 30). In order to investigate whether the sequestration of Cu(I) resulted in the restoration of trypsin activity, bathocuproine was used as a Cu(I) sequestering agent. When included in the reaction containing Cu(II), bathocuproine significantly inhibited the enzyme inactivation (Figure 31), confirming the involvement of Cu(I) in the photoilluminated damage to trypsin. (Control samples were run parallel to actual reaction that contained all the reaction components except BSA to check the release of peptide due to autolysis if any from the proflavine-treated enzyme).

Electrophoretic Analysis

The above results were further confirmed by SDS-PAGE. The photosensitized proflavine induced fragmentation of enzyme in a time dependent manner. The major 24 kDa band, decreased intensity with time (figure 32). Similar degradation of commercial trypsin has previously been reported by other workers (Finotti and Manete, 1994). Some aggregation of the protein was also observed with the slight increase in the intensity of the stained area above the main 24 kDa band. Fragmentation was found to be greater when proflavine-Cu(II) was used (Figure 32 lane e, f, g).

Involvement of various free radicals like O^1_2, O^3_2 and $^{\cdot}OH$ was tested by using the scavengers like sodium azide, potassium iodide and thiourea. They gave effective scavenging in the protein degradation reaction (Figure 33).

Fluorescent quenching studies on protein using proflavine

Most proteins exhibit ultraviolet fluorescence with emission of 340 nm due to the presence of tryptophan moiety. When a ligand binds to protein at or around tryptophan it may quench its native fluorescence (Chignell, 1972). BSA is known to bind to a variety of endogenous and exogenous ligands (Thiessen et al., 1972; Brodersien, 1974). Evidence for the binding of proflavine to proteins was obtained mainly from fluorescence spectra. We have compared the binding of proflavine with or without Cu(II) to BSA as well as with that of two other proteins having different

Figure 29. Trypsin inactivation as a function of increasing proflavine concentration.

Reaction mixture in a total volume of 2 ml contained 15 μg of trypsin, 10 mM sodium phosphate buffer pH 7.4 and increasing concentration of proflavine with or without 100 μM Cu(II). Reactions were incubated at 25°C for 35 minutes under fluorescent light. Enzyme activity was assayed as described in 'Methods'.

(-■-) Trypsin alone.

(-▲-) Trypsin + proflavine.

(-Δ-) Trypsin + proflavine + Cu(II).

(-●-) Trypsin + proflavine in dark.

(-○-) Trypsin + proflavine + Cu(II) in dark.

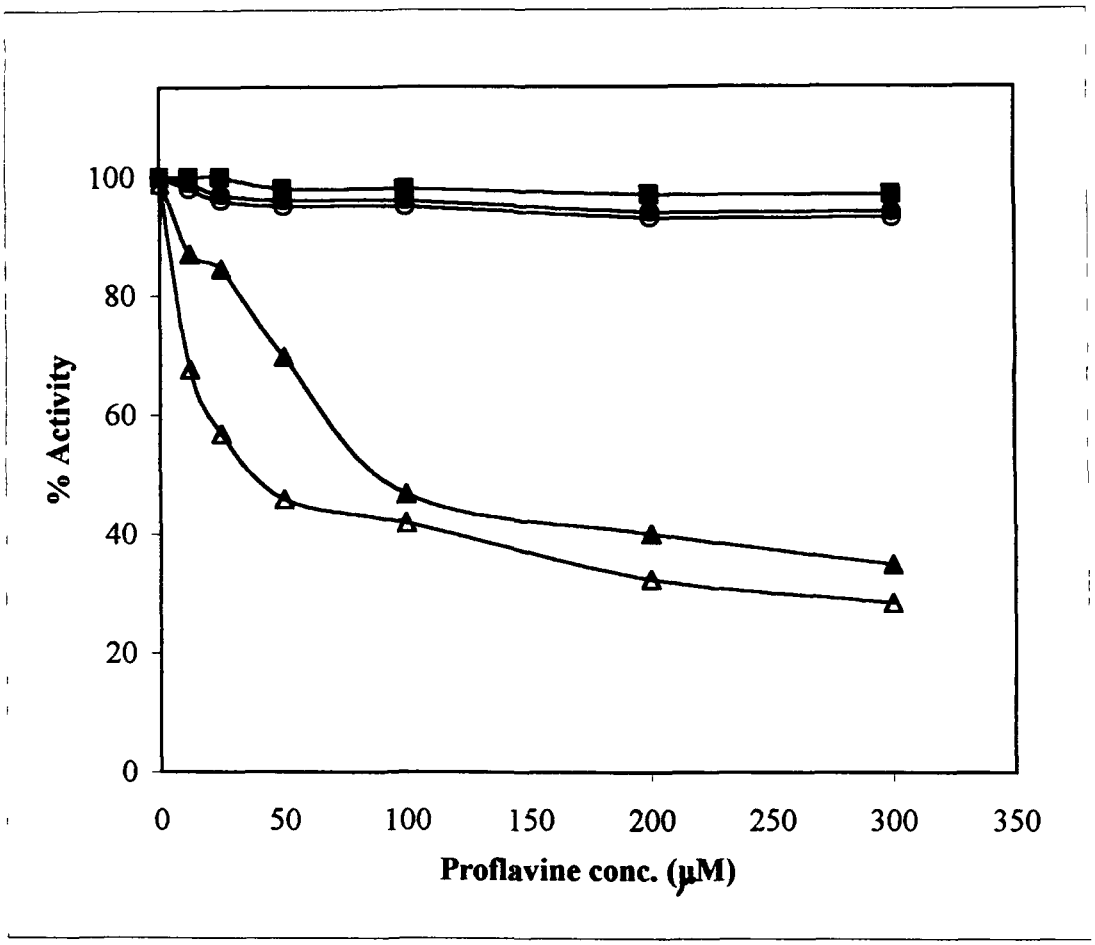

100
80
60
40
20
0
% Activity
0
50
100
150
200
250
300
350
Proflavine conc. (μM)

Figure 30. Trypsin inactivation by proflavine as a function of increasing Cu(II) concentration.

Reaction mixture in a total volume of 2 ml contained 15 μg of trypsin, 10 mM sodium phosphate buffer pH 7.4, 200 μM proflavine and increasing concentration of Cu(II). Reactions were incubated at 25°C for 35 minutes under fluorescent light. Enzyme activity was assayed as described in 'Methods'.

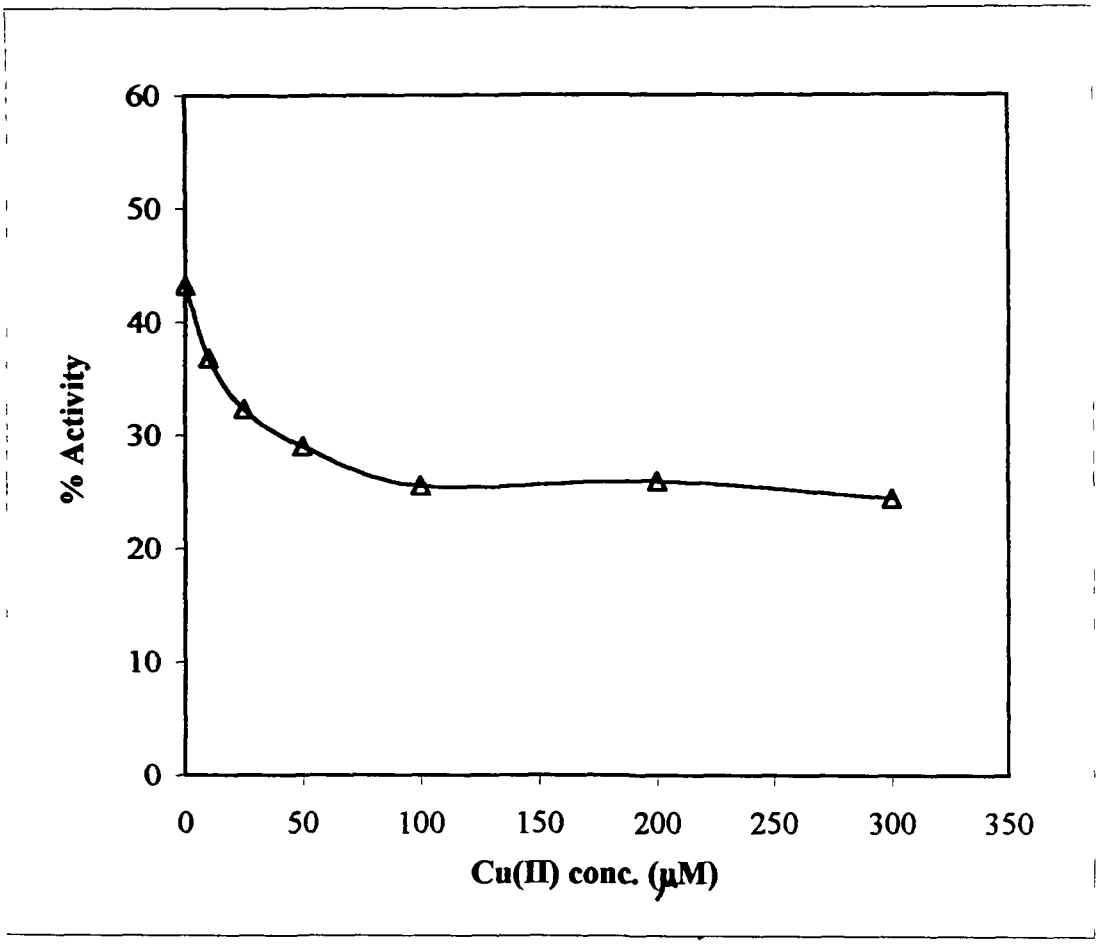

60
50
40
30
20
10
0
% Activity
0
50
100
150
200
250
300
350
Cu(II) conc. (µM)

Figure 31. Proflavine-Cu(II) induced inactivation of Trypsin: Inhibition by bathocuproine.

Reaction mixture in a total volume of 2 ml contained 15 μg of trypsin, 10 mM sodium phosphate buffer pH 7.4, 200 μM proflavine, 100 μM Cu(II) and increasing concentration of bathocuproine.

Reactions were incubated at 25°C for 35 minutes under fluorescent light. Enzyme activity was assayed as described in 'Methods'.

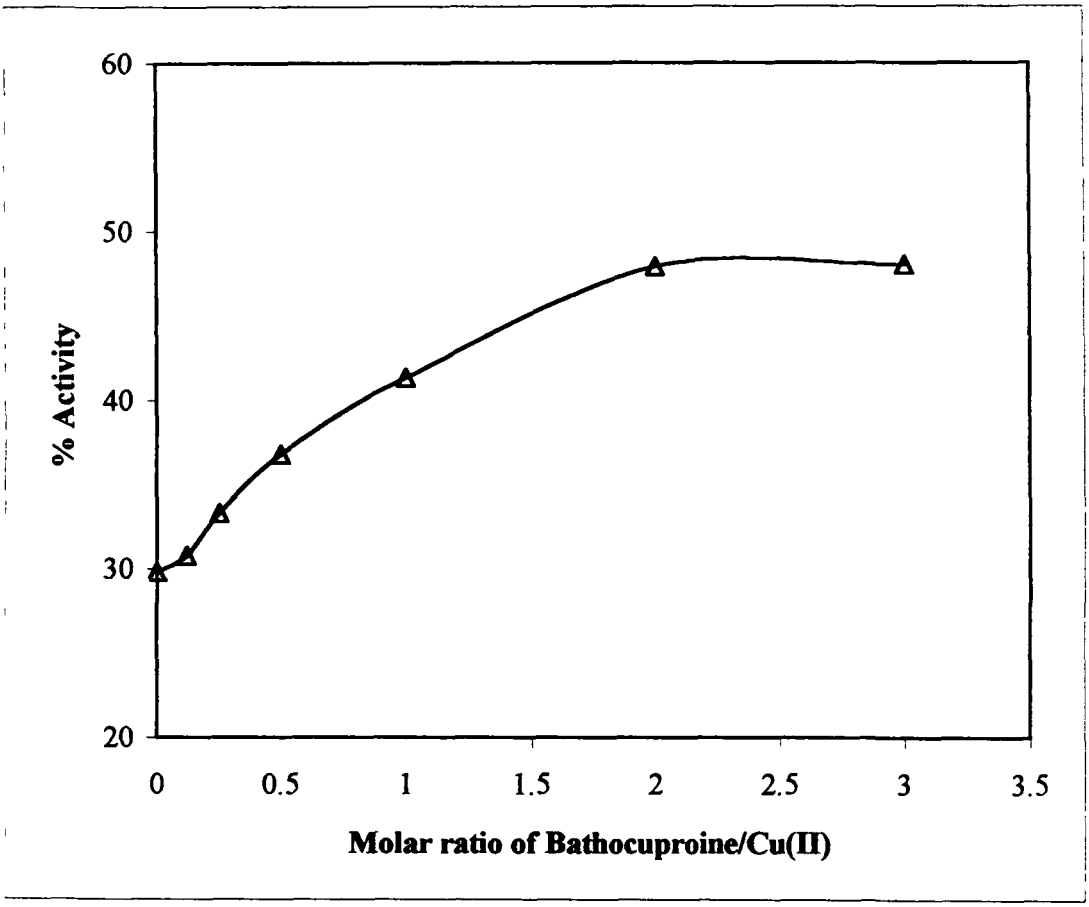

60
50
40
30
20
% Activity
0
0.5
1
1.5
2
2.5
3
3.5
Molar ratio of Bathocuproine/Cu(II)

Figure 32. Polyacrylamide gel electrophoresis of trypsin incubated with proflavine and Cu(II).

Electrophoresis was performed on 15% SDS-polyacrylamide gel as described under 'Methods' and 10 μg of protein was applied in each lane.

Lane a - Control (Trypsin incubated alone).

Lane b-d - Trypsin incubated for 4, 8 and 12 hours with proflavine.

Lane e-g - Trypsin incubated for 4, 8 and 12 hours with proflavine and Cu(II).

The reaction mixture (0.1 ml) contained 2 mg/ml trypsin (which was heat inactivated before addition to the reaction mixture), 10 mM sodium phosphate buffer (pH 7.4) and 200 μM proflavine and 100μM Cu(II). Incubation was carried out at 25°C for the indicated time intervals.

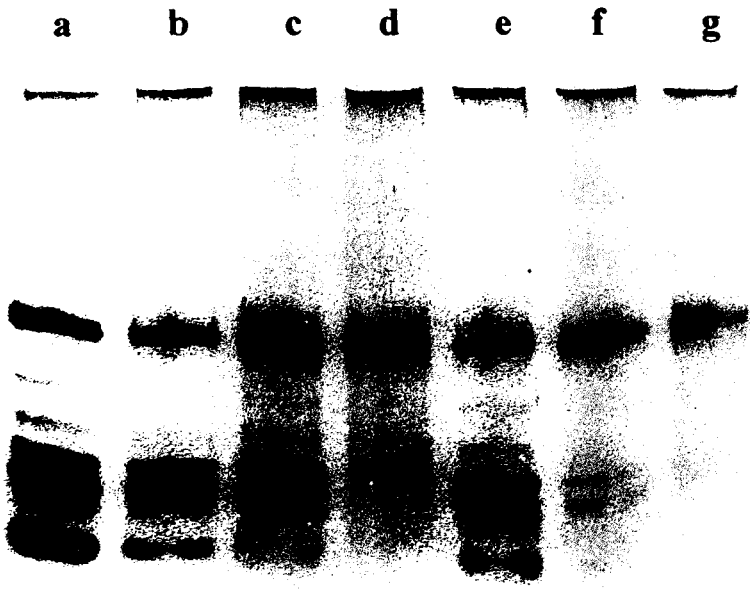

a
b
c
d
e
f
g

Figure 33. Proflavine induced degradation of trypsin. Inhibition by various radical scavengers.

Electrophoresis was performed on 15% SDS-polyacrylamide gel as described under 'Methods' and 10 μg of protein was applied in each lane.

(A) Trypsin incubated with 200 μM proflavine:

(lane a) Trypsin alone.

(lane b) Trypsin incubated with proflavine.

(lanes c, d, e) Trypsin, proflavine and 50 mM of sodium azide or potassium iodide or thiourea respectively.

(B) Trypsin incubated with 200 μM proflavine and 200 μM Cu(II):

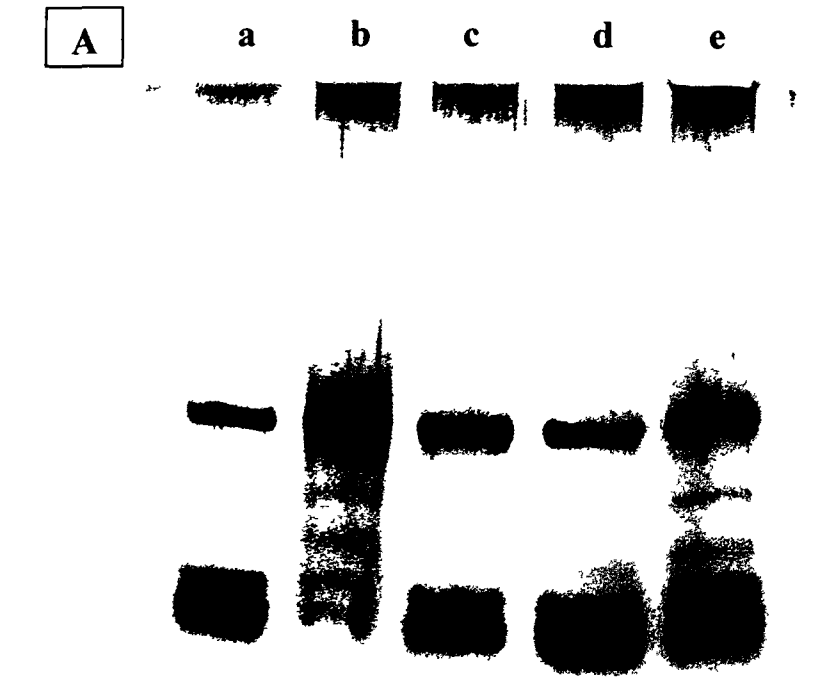
(lane a) Trypsin alone.

(lane b) Trypsin with proflavine.

(lane c) Trypsin with proflavine and Cu(II).

(lanes d, e, f) 50 mM of sodium azide or potassium iodide or thiourea respectively with trypsin, proflavine and Cu(II) system.

In both **(A)** and **(B)** the reactions were incubated for eight hours in fluorescent light at room temperature and gels were silver stained after electrophoresis.


A
a
b
c
d
e
B
a
b
c
d
e
f

tryptophan content (Table 2). The fluorescence quenching of BSA was measured with proflavine concentration in the range 0-20 μM (Figure 34). 52% quenching with 20 μM proflavine was observed. We suspected that proflavine binds to BSA on or near to tryptophan residues, we therefore, compared the binding of proflavine to BSA with tryptophan alone (Figure 35-B) and with two other proteins differing in tryptophan content, namely invertase (Figure 35-C) and lysozyme (Figure 35-D). All three proteins showed a shift of the λ_{max} for tryptophan fluorescence to lower wavelength with respect to the fluorescence of the free amino acid, it was giving 49%, 63% and 56% quenching for BSA, invertase and lysozyme respectively indicating increase in quenching with the increase of tryptophan residues of the protein. It is seen that in all cases the fluorescence quenching increases with increasing proflavine concentration. From a scatchard analysis of the data (Levine, 1977), the binding affinity of BSA was compared with that of the two other proteins i.e. lysozyme and invertase. The binding affinities of BSA (Figure 36), lysozyme (Figure 37) and invertase (Figure 38) were $11x10^{-4}$, $23\ x10^{-4}$ and $201x10^{-4}$ respectively. It was seen that the binding capacity of invertase was higher than that of the two other proteins, possibly reflecting the higher tryptophan content and that these residues are in hydrophobic environment (Campbell and Dwek, 1984).

We have also investigated the effect of proflavine with or without Cu(II) on the fluorescence spectra of trypsin. Figure 35-E showed a shift of the λ_{max} for fluorescence to lower wavelength with respect to the fluorescence of the free amino acid. Free tryptophan when mixed with proflavine or proflavine and Cu(II) (Figure 35-A) also conform these results showing 63% quenching in presence of proflavine. This confirms that BSA is an example of a possibly small subset of proteins to which proflavine can bind by mechanisms that affect fluorescence of tryptophan residues.

Table 2

Tryptophan residue concentration in some proteins.

Proteins	Number of Tryp. residues/protein	Reference
Bovine Serum Albumin	2	Ahmed et al ، 1994
Lysozyme	6	- do -
Invertase	33	Gascon et al ، 1968

Figure 34. Fluorescence emission spectra of bovine serum albumin (BSA) mixed with proflavine.

The 2.0 ml reaction mixture contained 10 mM sodium phosphate buffer, pH 7.4, 0.9 μM BSA. Increasing concentration of proflavine was added. BSA was excited at 280 nm and the emission spectra were recorded with emission slit of 10 nm.

Trace A - BSA alone.

Trace B- BSA + proflavine (1 μM)

Trace C- BSA + proflavine (6 μM)

Trace D- BSA + proflavine (10 μM)

Trace E- BSA + proflavine (20 μM)

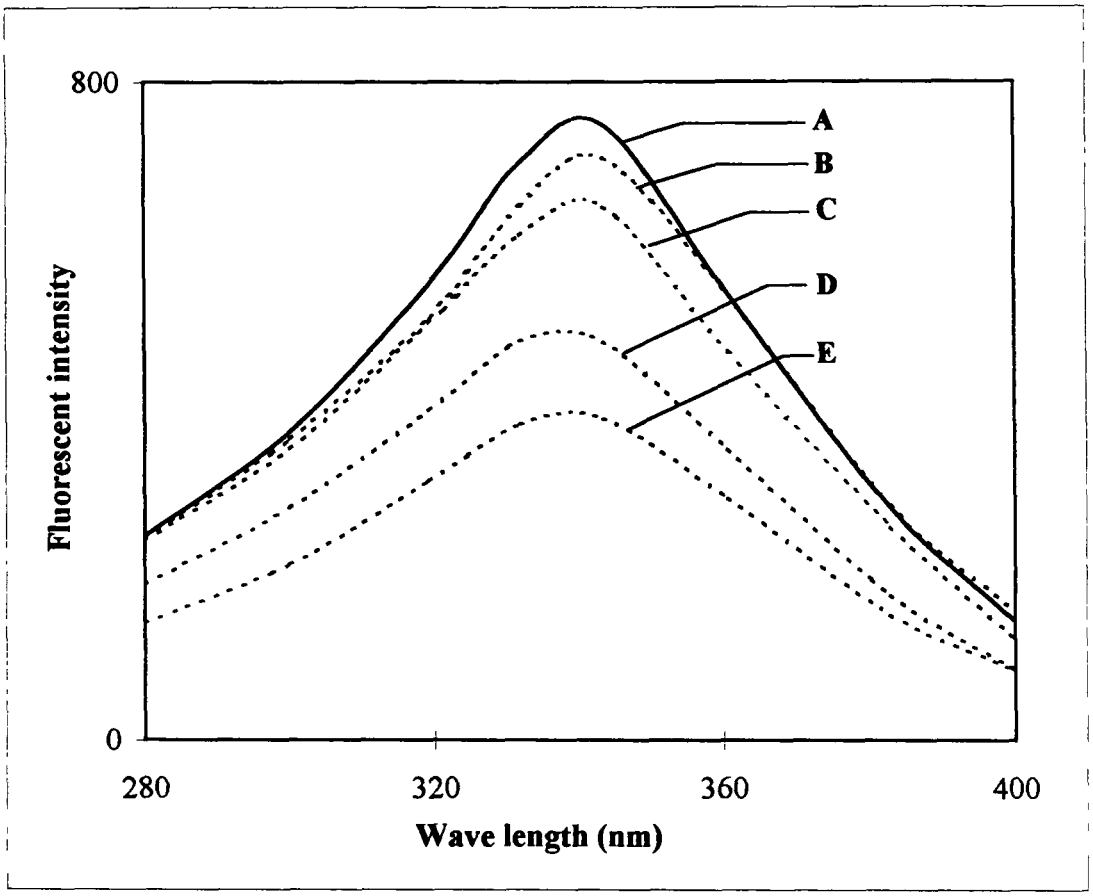

800
0
Fluorescent intensity
A
B
C
D
E
280
320
360
400
Wave length (nm)

Figure 35. Fluorescence emission spectra of different proteins with proflavine/proflavine-Cu(II).

The 2.0 ml reaction mixture contained 10 mM sodium phosphate buffer, pH 7.4, 0.7 μM protein (___), protein with Cu(II) (12.5 μM) (---), protein with proflavine (25 μM) (....), and protein with proflavine and Cu(II) (.._.._..). The excitation wave length was 280 nm and emission slit was 10 nm.

(A) Tryptophan

(B) BSA : blue shift from 380nm to 356nm with a weak peak.

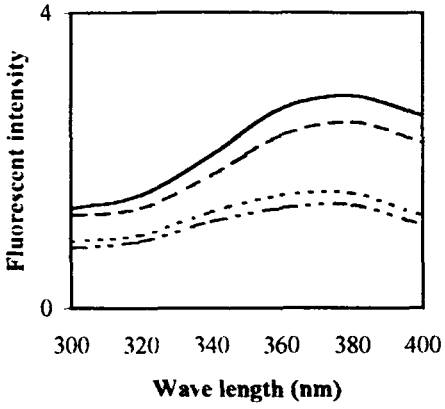
(C) Invertase : maximum blue shift from 380nm to 352nm with a clear peak.

(D) Lysozyme : blue shift from 380nm to 358nm with a broad peak.

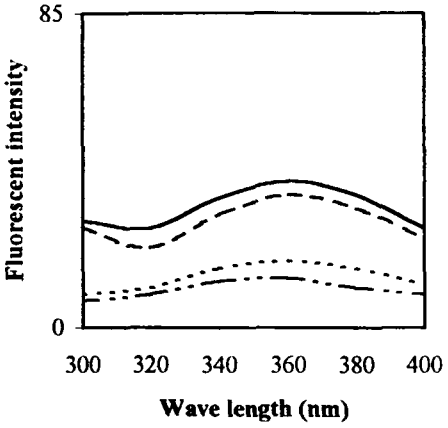
(E) Trypsin : blue shift from 380nm to 373nm with a broad peak.

All were compared to free tryptophan.

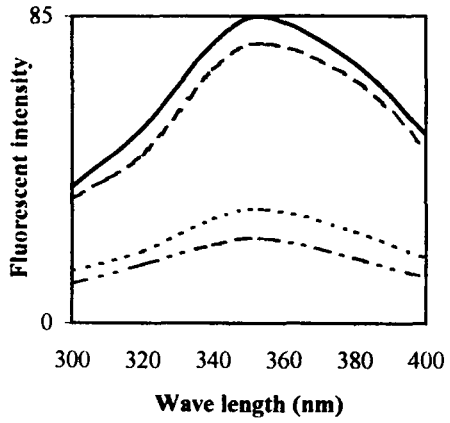
A


4
0
Fluorescent intensity
300 320 340 360 380 400
Wave length (nm)

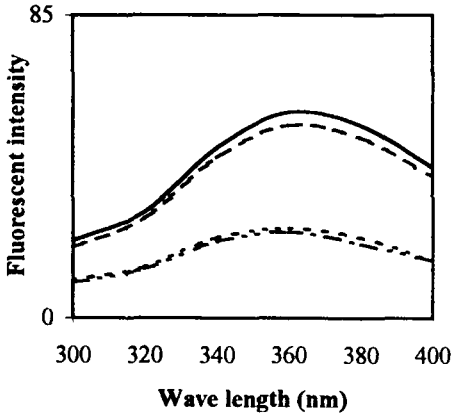
B


85
0
Fluorescent intensity
300 320 340 360 380 400
Wave length (nm)

C


85
0
Fluorescent intensity
300 320 340 360 380 400
Wave length (nm)

D


85
0
Fluorescent intensity
300 320 340 360 380 400
Wave length (nm)

E

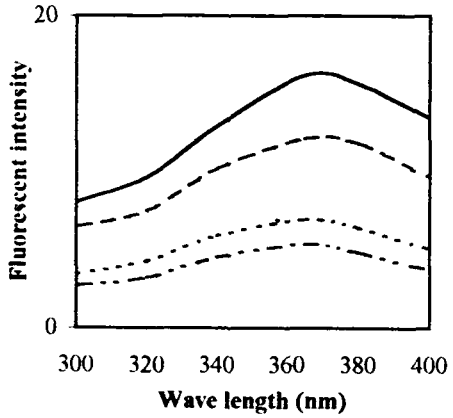

20
0
Fluorescent intensity
300 320 340 360 380 400
Wave length (nm)

Figure 36. Scatchard plot of BSA.

Scatchard plot was obtained using relationship of fractional quench (Q) with (Q/B), where B represents the concentration of unbound ligand (Levine, 1977).

(-▲-) BSA/Proflavine.

(-Δ-) BSA/Proflavine in presence of Cu(II).

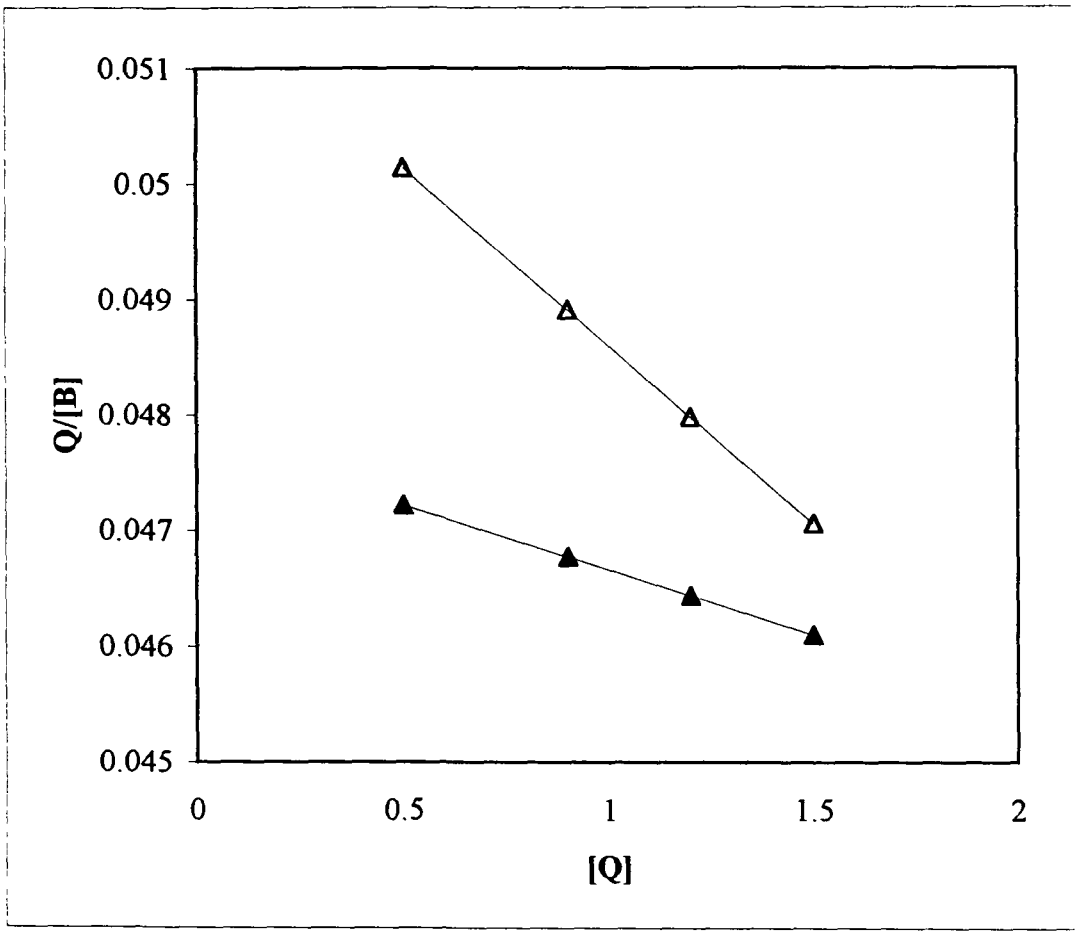

0.051
0.05
0.049
0.048
0.047
0.046
0.045
Q/[B]
0
0.5
1
1.5
2
[Q]

Figure 37. **Scatchard plot of Lysozyme**.

Scatchard plot was obtained using relationship of fractional quench (Q) with (Q/B), where B represents the concentration of unbound ligand (Levine, 1977).

(-▲-) Lysozyme/Proflavine.

(-Δ-) Lysozyme /Proflavine in presence of Cu(II).

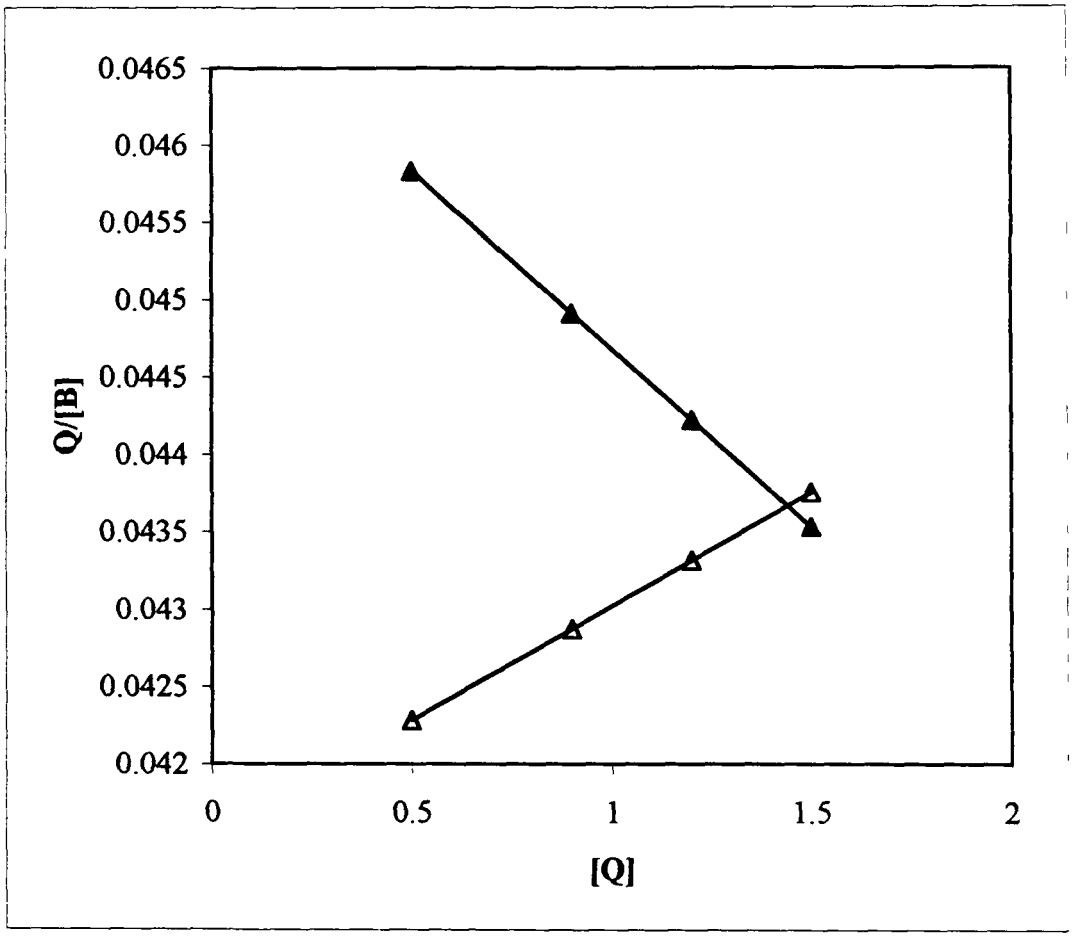
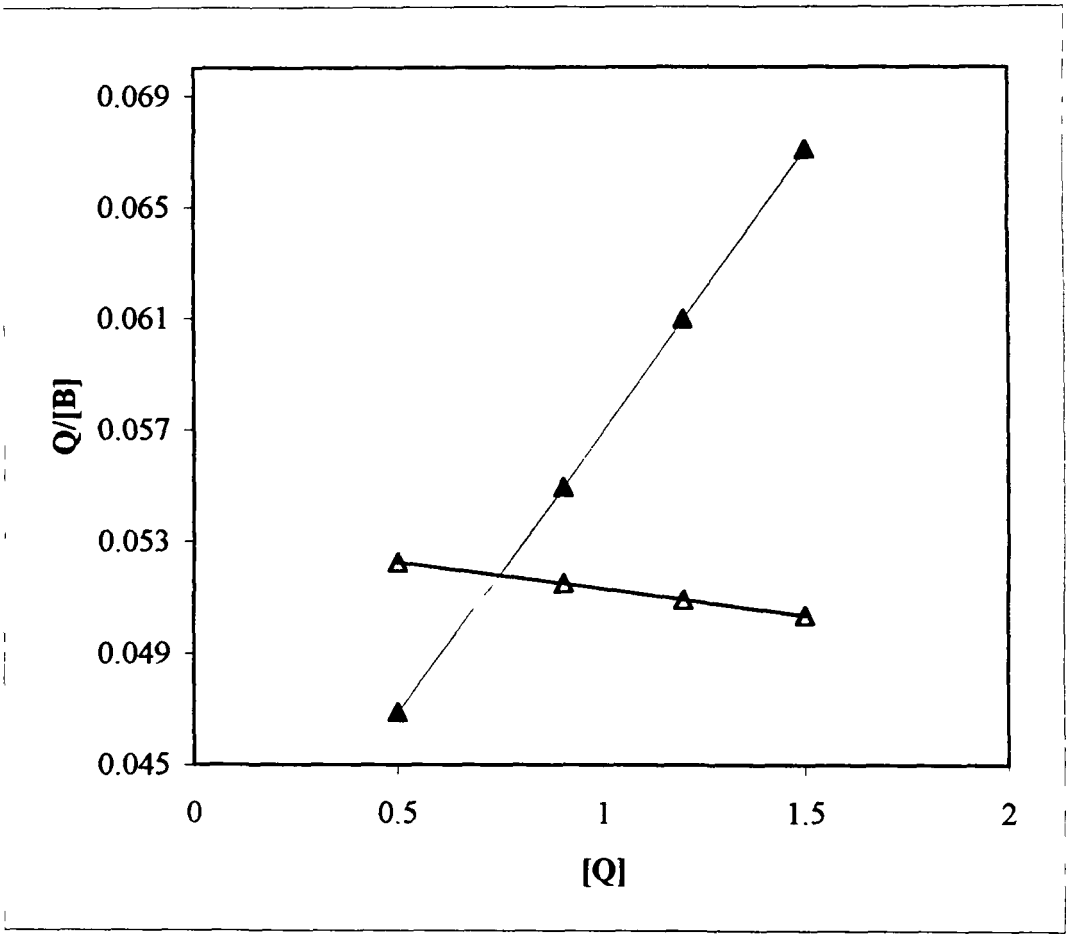

0.0465
0.046
0.0455
0.045
0.0445
0.044
0.0435
0.043
0.0425
0.042
Q/[B]
0
0.5
1
1.5
2
[Q]

Figure 38. Scatchard plot of Invertase.

Scatchard plot was obtained using relationship of fractional quench (Q) with (Q/B), where B represents the concentration of unbound ligand (Levine, 1977).

(-▲-) Invertase/Proflavine.

(-Δ-) Invertase /Proflavine in presence of Cu(II).


0.069
0.065
0.061
0.057
0.053
0.049
0.045
Q/[B]
0
0.5
1
1.5
2
[Q]

Discussion

Acridine dyes, especially proflavine, are photodynamic agents known to generate different types of reactive oxygen species upon illumination with visible light only when a macromolecule such as DNA is present in the reaction (Leupold and Kochevar, 1997). These reactive oxygen species are known to target DNA and other biomolecules (Leupold and Kochevar, 1997; Kochevar and Buckley, 1990). Although the exact mechanism of damage to DNA by proflavine is not well characterized, there are indications that proflavine is a type 1 photosensitizer in which its electronically excited state reacts directly with DNA (Piette *et al.*, 1981; Van de Vorst *et al.*, 1976). Both single- and double-strand breaks of plasmid DNA by photoilluminated proflavine have been observed (Shafirovich, 1999).

Our spectral studies indicate that upon photoillumination proflavine undergoes photodegradation and in the process reactive oxygen species are generated. Significantly, these reactive oxygen species are generated even in the absence of a macromolecule. This is in contrast to the observations of Piette and co-worker (Peitte *et al.*, 1978; Piette *et al.*, 1979; Piette *et al.*, 1981; Piette *et al.*, 1982) who found that the reactive oxygen species are generated upon photoillumination of proflavine but only when a macromolecule like DNA is also present in the reaction (Kochevar and Dunn, 1990). The addition of sodium azide, potassium iodide and thiourea significantly inhibited the photodegradation of proflavine. The effect is most pronounced with potassium iodide indicating the O_2^3 is the major species generated in the reaction. The spectral studies also indicate that the presence of Cu (II) during photoillumination of proflavine partially restores the proflavine peak, but does not causes enhancement in the production of ROS.

The formation of $^{\cdot}OOH$ and $^{\cdot}OH$ during the DNA strand breakage reaction have been proposed by Piette et al. (1982). According to their proposed mechanism, proflavine may also give rise to $O_2^{\cdot-}$ either by direct reaction between the electron ejected by excited proflavine and molecular oxygen or by the decomposition of $^{\cdot}OOH$. We have shown that around 55% inhibition to NBT reduction reaction occurred when SOD was present in the reaction confirming the formation of $O_2^{\cdot-}$ in addition to other reactive oxygen species in the reaction. However, several authors have implicated a highly reactive $^{\cdot}OH$ derived from $O_2^{\cdot-}$ as the ultimate reactive species (Fridovich, 1978; Halliwell *et al.*, 1980; Halliwell, 1981). The $O_2^{\cdot-}$ and H_2O_2 can generate $^{\cdot}OH$ by either

modified Haber-Weiss (1) or by Fenton (2) reactions provided a metal ion is present in the latter reaction.

$$O_2^{\cdot-} + H_2O_2 \rightarrow {}^{\cdot}OH / {}^{-}OH + O_2 \quad (1)$$

$$H_2O_2 + Fe(II)/Cu(I) \rightarrow {}^{\cdot}OH + {}^{-}OH + Fe(III)/Cu(II) \quad (2)$$

It is also known that the generation of the $O_2^{\cdot-}$ may lead to the formation of H_2O_2. The addition of a second electron to $O_2^{\cdot-}$ gives the peroxide anion ($O_2^{\cdot-2}$) which has no unpaired electron and is not a radical. However, at neutral pH the peroxide ion immediately gets protonated to give H_2O_2. Alternatively, in aqueous solution the $O_2^{\cdot-}$ undergoes dismutation to form H_2O_2 and O_2 (Halliwell and Gutteridge, 1984):

$$2O_2^{\cdot-} + 2H \rightarrow H_2O_2 + O_2 \quad (3)$$

We have shown through the deoxyribose reaction the ability of proflavine to generate ·OH alone as well as in presence of Cu(II) supporting the finding of Piette and co-workers (Piette *et al.*, 1981; Piette *et al.*, 1982). However, in contrast to their observations the ·OH is generated even in the absence of DNA.

Most of the study that have been carried out by Piette and co-workers (Peitte *et al.*, 1978; Piette *et al.*, 1979; Piette *et al.*, 1981; Piette *et al.*, 1982) have shown that induction of radical is possible only in the presence of macromolecule as DNA and this was reported to induce the formation of peroxide radical and singlet oxygen. Van de Vorst et al. (1976) however, have suggested the involvement of superoxide and formyl radical in the same photoilluminated reaction mixture. Our observation indicates that the photogeneration of $O_2^{\cdot-}$ from proflavine was faster in presence of dsDNA. While both ssDNA and RNA were actually inhibitory. The spectral studies of proflavine in presence of these molecules suggest that the presence of ds DNA in the reaction helps in restoring the structure of proflavine more than RNA. This is probably due to the fact that proflavine intercalates with ds DNA. Radical produced in the process probably attack the DNA in the vicinity of proflavine binding before they can be detected. It is well known that intercalation of planar ligands between DNA base pairs involves partial unwinding of the double helix (Waring, 1970). Therefore, interstrand covalent crosslinks were expected to block intercalation. In the cross-linked dsDNA the stimulatory effect on $O_2^{\cdot-}$ formation was not observed. Availability of guanine residues

may be still necessary for the stimulatory effect of dsDNA on the photogeneration of $O_2^{\cdot-}$. Comparative observation using alkylated and depurinated DNA on the generation of $O_2^{\cdot-}$ by proflavine has clearly shown the better stimulatory effect of alkylated DNA than that of depurinated DNA. This result indicates that the presence of guanine residues in DNA is essential for the stimulatory effect of dsDNA on $O_2^{\cdot-}$ generation of by proflavine in visible light. This again supported the idea of Piette et al. (1978) who showed that intercalation of acridine dyes in DNA under light treatment favours the formation of free radicals.

A survey of literature revealed that interaction of proteins with oxygen free radicals both in vivo and in vitro results in enhanced hydrophobicity due to modifications of amino acids, hence, possible increase in the susceptibility to proteolysis (Wolff and Dean, 1986; Davies and Doroshow, 1986; Davies *et al.*, 1987). Wolff and Dean (1986) have shown that ·OH attack on proteins leads to conformational changes and therefore, enhanced susceptibility to enzymatic proteolysis. It is well reported that limited protein oxidation can increase hydrophilicity while further oxidation by radical attack can increase hydrophobicity (Kim *et al.*, 1985). In our observation however, susceptibility to tryptic proteolysis of BSA was decreased in presence of proflavine and Cu(II) indicating the possibility of modification of specific amino acids, lysine and arginine which are the amino acids residues that are recognized by trypsin. Others have reported that these amino acids are modified after radical attack (Kang *et al.*, 1985), supporting our observation.

BSA is known to bind to a variety of endogenous and exogenous ligands (Thiessen et al., 1972; Brodersen, 1974). When a ligand binds to protein at or around tryptophan it quenches its native fluorescence (Chignell, 1972). The quenching of fluorescence of tryptophan residues is diagnostic of the binding of ligand to a tryptophan or alternatively of a conformational change caused by interaction (Levine, 1977). BSA and three other proteins differing in their tryptophan content, namely lysozyme, invertase and trypsin, have been used for fluorescence studies. A shift in λ_{max} for tryptophan fluorescence together with quenching was observed in all the cases, suggesting the binding of proflavine to these proteins in the vicinity of tryptophan residues. The shift in λ_{max} also suggests that these tryptophan residues are in hydrophobic environment (Ahmed et al., 1994). This is consistent with at least the

known crystallographic structure of lysozyme. The shift in λ_{max} was maximum with invertase followed by BSA and lysozyme and minimum with trypsin. This suggests that the tryptophan of trypsin is in the least hydrophobic environment.

As the pattern of fluorescence quenching was not fully in accordance with the number of tryptophan residues in the proteins, it is therefore proposed that proflavine being hydrophobic in nature shows binding characteristics to BSA similar to quercetin (Ahmed et al., 1994). Scatchard analysis of the data showed that the proflavine binding capacity was in the order of invertase > trypsin > lysozyme > BSA, reflecting the different tryptophan content (Campbell and Dwek, 1984). The emission spectra of BSA recorded after addition of increasing proflavine concentrations also indicate that proflavine either binds to tryptophan directly or modifies the conformation of BSA, leading to shielding of tryptophan as evidenced by the progressive decrease in emission intensity.

Proteins are the key targets of reactive oxygen species leading to their oxidation which may sometimes control cellular remodeling and growth. (Dean *et al.*, 1997). Protein fragmentation has previously been reported following exposure to $^{\cdot}OH$ and $O_2^{\cdot-}$ in presence of oxygen. The fragmentation process was suggested to involve hydrogen abstraction by $^{\cdot}OH$ from amino acid α-carbon atoms, followed by reaction with O_2 to produce peroxyl species oxygen. Decomposition of α–carbon peroxides was proposed as the mechanism for protein fragmentation oxygen (Garrison *et al.*, 1962). Davies and Delsignore (1987) have shown fragmentation of BSA upon exposure to $^{\cdot}OH$ and $O_2^{\cdot-}$ under aerobic condition which is broadly consistent with the above proposed mechanism. We, therefore, studied the effect of reactive oxygen species generated upon photoillumination of proflavine on BSA. Proflavine alone upon photoillumination, caused pronounced degradation of BSA. The addition of Cu(II) in the reaction significantly enhanced this degradation. Spin trapping technique provided evidence for the production of $^{\cdot}OH$ during proflavine mediated degradation of DNA (Piette *et al.*, 1982). We have shown that $^{\cdot}OH$ are involved in degradation of BSA by photoilluminated proflavine since the addition of thiourea (a scavenger of $^{\cdot}OH$) to either proflavine or proflavine-Cu(II) significantly inhibited this degradation. However, there is strong evidence for the formation of O^1_2, O^3_2 also as sodium azide and potassium iodide also inhibited protein degradation reactions to a significant extent. This was

further confirmed using two other enzymes with different structure properties. First was a glycoprotein, namely invertase, which was also fragmented by proflavine alone and the presence of Cu(II), enhanced this fragmentation as revealed in SDS-PAGE. However, in case of monomeric nonglycoprotein, trypsin, proflavine led to intramolecular crosslinking, while the presence of Cu(II) significantly decreased the extent of crosslinking. Our results are comparable to the finding of Sara et al. (1984) who have suggested that multimeric proteins when exposed to free radical generating systems show visible fragmentation, while crosslinking was predominant in case of monomeric proteins. The work carried out by Davies and Delsignore (1987) points to the production of bityrosine upon exposure to $^{\cdot}OH$ and $O_2^{\cdot-}$ which result in late degradation of the protein with the formation of aggregate. But in case of glycosylated protein, the fragmentation induced by proflavine alone is slow which could be due to the presence of attached carbohydrates. However, Wollf and co-workers (Wolff and Dean, 1987; Wolff *et al.*, 1989) have proposed that the presence of Cu(II) could catalyse the auto-oxidation and hence the fragmentation of the protein is expected in a system that generates $^{\cdot}OH$.

Proflavine has been reported to form hydrogen bonds with some amino acids with one of its amino groups or with the protonated ring nitrogen (Conti *et al.*, 1998). We have shown the evidence for the binding of proflavine to proteins through fluorescence quenching studies. The fluorescence of proteins decreased with increasing proflavine concentration. Decreased fluorescence reflects changes in the aromatic side-chain composition, possibly a loss of conjugation from tryptophan or other aromatic amino acids (Gutteridge and Wilkins, 1983).

We have used copper in our studies since it is an essential trace element that is distributed throughout the body. It is present in many tissues including liver and kidney where its concentration is relatively high (Burkitt *et al.*, 1996). Copper forms the essential redox-active centre in a variety of metalloproteins such as ceruloplasmin, Cu,Zn superoxide dismutase, cytochrome c oxidase, tyrosinase and ascorbate oxidase. Normal serum contains up to 8 μM loosely bound copper which is available for redox reaction (Bukitt *et al.*, 1996; Rowley and Halliwell, 1983). When bathocuproine, a Cu(I) sequestering agent, was used in the degradation reaction of BSA that contains Cu(II) it has given significant inhibition showing the involvement of Cu(I). Thus it appears that

binding of proflavine leads to formation of proflavine-protein-Cu(II) complex, which upon photoexcitation via charge transfer is converted into oxidized proflavine-protein-Cu(I), resulting in generation of reactive oxygen species which then mediate protein fragmentation.

The results of our work with proteins showed that photoilluminated proflavine caused significant structural and functional alterations. The accumulation of catalytically inactive or less active, more heat labile forms of many enzymes is an index of cellular aging (Dreyfus *et al.*, 1978; Rothstein, 1977). Invertase catalyses the hydrolysis of sucrose to mixture of glucose and fructose or the so called invert sugar. The enzyme is of considerable industrial significance especially in the production of fructose containing syrups. Yeast invertase is a glycoprotein with about half its mass contributed by the high mannose type of the oligosaccharide chains (Trimble and Maley, 1977). Most of the glycoenzymes (Lis and Sharon, 1993) including invertase (Lehle *et al.*, 1979) do not require their glycosyl residues for their catalytic functions. The presence of ogligosaccharides did not protect the enzyme from attack by radicals which were generated from the photoilluminated proflavine. The loss of activity was due to proflavine derived free radical mediated damage to protein. Metal-catalysed oxidation systems are reported to inactivate a wide range of enzymes (Bauskin *et al.*, 1991; Stadtman, 1991), and invertase is known to bind to Cu(II), hence, the presence of Cu(II) in the reaction is expected to enhance the degradation of protein. Presence of Cu(II) in the reaction mixture resulted in enhanced degradation of protein to smaller peptide. This is in agreement with the finding that radical attack on glycoproteins stimulates peptidolysis (Cooper *et al.*, 1985). Wolff and co-worker (Wolff and Dean, 1987; Wolff et al., 1989; Hunt *et al.*, 1988) have shown that hydroxyl radical and Cu(II) also catalyze the autoxidation of sugars which can then participate in the modification of proteins.

Proflavine is known to bind competitively to trypsin (Brantner *et al.*, 1976) and prevents substrate binding. The rate of the formation of enzyme-inhibitor (proflavine) complex is equal to the rate of formation of enzyme substrate complex, hence high concentration of substrate is required for the formation of enzyme substrate complex when an inhibitor like proflavine is also present in the reaction. BSA the substrate for trypsin in our study, was always used in excess for this reason. Even so, the proteolytic activity of trypsin was shown to be markedly decreased, probably due to free radical

mediated damage to specific amino acids in the protein by proflavine. The loss of activity of trypsin was more pronounced when Cu(II) was also included in the reaction. The degradation of BSA by proflavine was higher than trypsin as shown by SDS-PAGE analysis. Same observation was recorded by other investigators. After exposing trypsin to ·OH (Davies, 1987) no polypeptide fragmentation was observed; rather intramolecular bi-tyrosine was produced by reaction of two tyrosyl radicals or a tyrosyl radical plus a tyrosine molecule (Prutz *et al.*, 1983). As the trypsin degradation following exposure to proflavine or proflavine and Cu(II) was not very significant, this could also be due to the production of intramolecular bi-tyrosine, as the band intensity or migration is not much effected on SDS-PAGE.

Glycopolypeptides are effective scavengers of ·OH when they are generated in the system. Such scavenging action is expected from the high sugar content of the glycopolypeptides. This scavenging action may be of primary importance in protecting the respiratory and gastrointestinal tracts from adventitious radicals in smoke or those produced by normal cellular action (Cross *et al.*, 1984). Other reports have shown that peroxide radical attack on glycoproteins occurs largely at the histidine residues causing its modification (Cooper *et al.*, 1985). Wolff and co-workers (Wolff and Dean, 1987; Wolff *et al.*, 1989; Hunt *et al.*, 1988) have proposed that hydroxyl radical and Cu(II) catalyze auto-oxidation of the sugars which can then participate in the modification of protein. Modification of proteins by radical attack have been previously reported by Gantchev and Van Lier (1995) where they have shown that radical generated by photosensitizer like tetrasulfonated metasllophthalocyanines can change the activity of catalase. It also caused conformational changes in the enzyme. Singlet oxygen and other radical species can participate in the photosensitizer induced inactivation of enzymes as exemplified by other studies on purified catalase, or catalase within cells (Gantchev and Van Lier, 1995). Our studies have supported the above mentioned report of Cross et al. (1984) for our target enzyme, the heavily glycosylated invertase, which has shown a 43% decrease of activity after five hours of exposure to proflavine and light. This resistance could be due to the presence of carbohydrates on the surface of the enzyme which upon reacting with various reactive oxygen species produced by proflavine may themselves participate in the scavenging of ·OH produced in the system. The enzyme has shown enhanced inactivation in presence of proflavine and Cu(II), such decrease in the activity could be due to the attached carbohydrate. Invertase can bind to Cu(II)

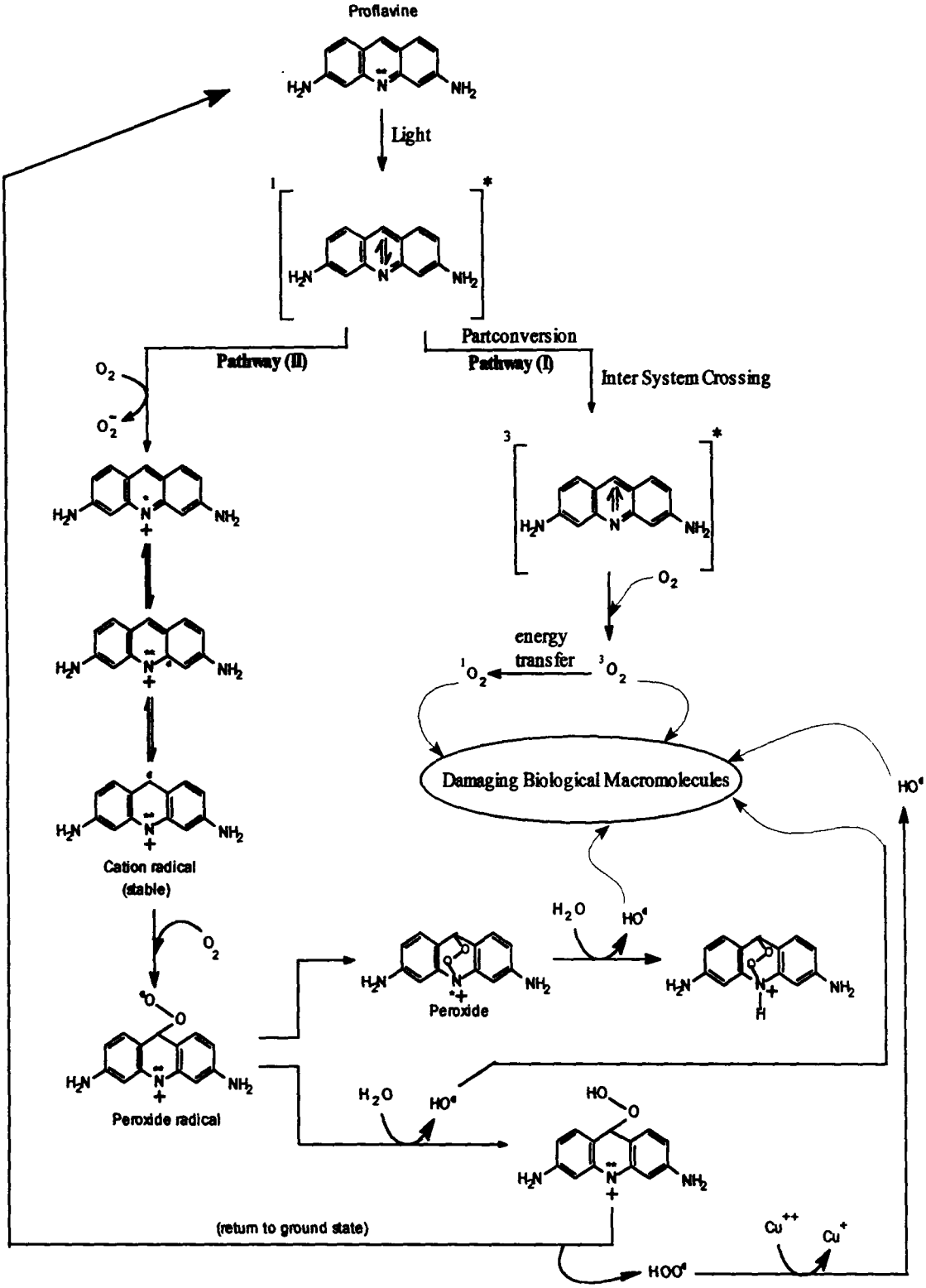
directly and the activity of invertase is inhibited, or the presence of bound Cu(II) directed the reactive oxygen species generated in the reaction to target the active site, thereby, inhibiting the enzyme (Bernfeld, 1955). This was confirmed by the use of metal chealating agent like EDTA. The inactivation of the enzyme was slow in the presence of EDTA. Comparing this study with the non glycosylated enzyme trypsin we have shown that after photoillumination with same system for only twenty minutes trypsin showed 40% residual activity, and was completely inactivated after one hour of incubation with proflavine and light. The enzyme seems to be directly effected by the ROS generated from proflavine or proflavine and Cu(II). The effect being slightly more but not significant when Cu(II) is present. Trypsin has shown resistance to degradation on SDS-PAGE under similar condition compared to BSA probably for different reasons, which supports the observation of Davies (1987) suggesting the formation of bi-tyrosine crosslinks as discussed earlier.

From our studies on proflavine we propose the following probable mechanism for the generation of various reactive oxygen species from photoexcited proflavine (Figure 39). Proflavine upon photoillumination is excited to singlet state which gives rise to triplet state through inter system crossing. When H_2O and O_2 are present in the reaction photoexcited proflavine can then give rise to O_2^3 and O_2^1 through direct energy transfer (pathway (I)). These O_2^3 and O_2^1 can participate in the protein degradation reaction. Through an alternative pathway (II), the photoexcited proflavine can accept an electron from molecular oxygen and give rise to cationic radical which further reacts with molecular oxygen and give peroxide radical. This peroxide radical can, in the presence of H_2O, give $^{\cdot}OH$ or $^{\cdot}OOH$ and in the process Cu(II) may be reduced to Cu(I) if present in the reaction, and proflavine then returns to the ground state. Polyaromatic systems are well known to go for facile photoxidation in presence of light and oxygen to give peroxide radical. Furthermore, photochemical electron transfer reactions between transition metal ions and anions or water molecules are well established (Spinks *et al.*, 1964), moreover, exposure of water to pulse of electrons can cause ionization and excitation within 10^{-16} second (Halliwell and Gutteridge, 1985) as seen in the following example:

$$H_2O \rightarrow H_2O^+ + e^- + H_2O^* \qquad (1)$$

$$H_2O^* \rightarrow H^{\cdot} + {}^{\cdot}OH \qquad (2)$$

Figure 39. Scheme for the production of free radicals from the photoilluminated proflavine.


Proflavine
Light
Partconversion
Pathway (II)
Pathway (I)
Inter System Crossing
energy
transfer
Damaging Biological Macromolecules
Cation radical
(stable)
Peroxide
Peroxide radical
(return to ground state)

Where is H_2O^* represent an excited water molecule. And within the same timescale H_2O^+ also react to give ·OH

$$H_2O^+ + H_2O \rightarrow H_3O^+ + \cdot OH \qquad (2)$$

The ·OH and ·OOH, in addition to O^3_2 and O^1_2, are also available in the reaction to attack the target molecule and cause further damage.

The above study gains significance because proflavine is structurally similar to tacrine and quinacrine, drugs approved for the treatment of Alzheimer's disease and malaria, respectively (Plymale and de la Iglesia, 1999; Motten et al., 1999). Studies using human hepatocytes with tacrine and proflavine show subcellular changes and mitochondrial dysfunction (Plymale and de la Iglesia, 1999). Proflavine and 7-hydroxy tacrine have been implicated as potential precursors of reactive metabolites (Madden et al., 1995). A toxic role of oxidized proteins, rather than oxidized lipids, has been recently proposed in the etiology of Alzheimer's disease (Sigman et al., 1979). As tacrine is known to be converted to 7-hydroxy tacrine, the risk of protein oxidation leading to precipitation of disease will increase. Moreover, the ability of proflavine to inactivate certain types of miro-organisms can give possible use of photosterilization against contamination with pathogenic bacteria in water model ecosystmes, Kussoviski et al. (2001) have studied the survival of Salmonella dublin and heterotrophic bacteria in freshwater microcosms after exposing them to proflavine and sun light.

In conclusion, the data presented here confirm that proflavine has a photosensitizing action and in the presence of visible light generates various reactive oxygen species which can attack proteins leading to modification. The effect was enhanced in the presence of Cu(II). Further studies using in vivo system are, however, needed to confirm the damaging effect of proflavine.

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